

Unveiling gene expression and epigenetic regulators across human oocyte maturation

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UNVEILING GENE EXPRESSION AND EPIGENETIC REGULATORS ACROSS HUMAN OOCYTE MATURATION

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“Temos, sobretudo, de aprender duas coisas: aprender o extraordinário que é o mundo e aprender a ser bastante largo por dentro, para o mundo todo poder entrar.”

Agostinho da Silva

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PUBLICATIONS

This doctoral thesis is primarily based on a scientific article already published, titled "Transcriptomic analysis and epigenetic regulators in human oocytes at different stages of oocyte meiotic maturation." This study provides a comprehensive analysis of transcriptomic changes and key epigenetic regulators during oocyte maturation, shedding light on the molecular mechanisms underlying this critical process.

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In addition to the published work, this thesis includes original, unpublished results from immunofluorescence analyses of 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), TET3 and DNMT1 in oocytes at different maturation stages.

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ABBREVIATIONS

3'-UTR:	3'-Untranslated region
5caC:	5-carboxylcytosine
5fC:	5-formylcytosine
5hmC:	5-hydroxymethylcytosine
5mC:	5-methylcytosine
AC:	Adenyl cyclase
APC:	Anaphase-promoting complex
ATP:	Adenosine triphosphate (energy molecule)
cAMP:	Cyclic adenosine monophosphate
CC:	Cumulus cells
CG:	Cortical granules
CGI:	CpG islands
cGMP:	Cyclic guanosine monophosphate
COC:	Cumulus-oocyte complex
CPE:	Cytoplasmic polyadenylation element
CPEB:	CPE-binding protein
CpG:	Cytosine-guanine dinucleotide
CPM:	Counts Per Million
CPSF:	Cleavage and polyadenylation specificity factor
DEG:	Differentially expressed genes
DNMT:	DNA methyltransferase
DSB:	Double-strand break
EGA:	Embryonic genome activation
ER:	Endoplasmic reticulum
FC:	Fold Change
FDR:	False Discovery Rate
FSH:	Follicle-stimulating hormone
GA:	Golgi apparatus
GC:	Granulosa cells
gDMR:	Germline differentially methylated domains
GnRH:	Gonadotropin-Releasing Hormone
GO:	Gene Ontology
GV:	Germinal vesicle
GVBD:	Germinal vesicle breakdown
IP3:	Inositol trisphosphate

LH: Luteinizing hormone
LHR: Luteinizing hormone receptor
m5C: RNA methyl-5-cytosine
m6A: RNA N6 methyladenosine
MAPK: Mitogen-activated protein kinase
MGC: Mural granulosa cells
MI: Metaphase II oocyte
MII: Metaphase II oocyte
MPF: Maturation-promoting factor
mRNA- Messenger RNA
mtDNA: Mitochondrial DNA
NGO: Non-growing oocyte
NPPC: Natriuretic peptide C
NPR2: NPPC receptor
OSFs: Oocyte-secreted factors
PABPC: Cytoplasmic poly(A)-binding protein
PARN: Poly(A) ribonuclease
PAS: Poly(A) signal
PCA: Principal Component Analysis
PDE: Phosphodiesterase
PGCs: Primordial germ cells
qRT-PCR: Quantitative Reverse Transcription PCR
RER: Rough endoplasmic reticulum
rFSH: Recombinant Follicle-Stimulating Hormone
SC: Synaptonemal complex
SER: Smooth endoplasmic reticulum
TCs: Theca cells
TET: Ten-eleven translocation protein
TZPs: Transzonal projections
ZP: Zona pellucida

ABSTRACT

Human oocyte meiotic maturation is a critical process for successful fertilization and embryonic development. During the transition from the germinal vesicle (GV) stage to the metaphase II (MII) stage, significant nuclear and cytoplasmic changes occur, enabling the oocyte to achieve the competence required for fertilization by sperm. These transformations are influenced by dynamic shifts in gene expression and epigenetic regulation, which coordinate the activation, silencing, and storage of essential transcripts.

Therefore, understanding the interplay between transcriptomic and epigenetic regulation in oocytes is crucial for elucidating the molecular determinants of oocyte quality. These insights hold significant implications for improving assisted reproductive technologies (ART), particularly in optimizing in vitro maturation protocols and identifying biomarkers for oocyte viability. However, the human oocyte remains understudied compared to other mammalian species, and the specific mechanisms underpinning its maturation and developmental potential require further exploration.

In our study we aimed to fill these knowledge gaps by providing a detailed characterization of the transcriptomic changes of human oocytes in different stages of maturation (GV, MI and MII oocytes). Additionally, we also evaluated some epigenetics modifications and epigenetics regulators in different maturation stages of oocytes.

To accomplish that, we performed single-oocyte transcriptomic analysis of human oocytes in the three meiotic maturation stages, and we analysed oocytes stained with 5 methylcytosine(5mC), 5 hydroxymethylcytosine (5hmC), DNMT1 and TET3 antibodies. Single-oocyte transcriptomic analysis revealed a progressive decline in the total number of expressed genes as oocytes matured from the GV to the MII stage. Specifically, 9660 genes were transcribed in GV-stage oocytes, decreasing to 8734 in MI and 5889 in MII. The same tendency was observed for the number of uniquely expressed genes.

The analysis of differentially expressed genes (DEGs) between maturation stages identified 1165 DEGs between GV and MII oocytes, with 635 upregulated and 528 downregulated. Between GV and MI, only 42 DEGs were observed, of which 38 were upregulated and 4 downregulated, while no significant changes in gene expression were detected between MI and MII. A comprehensive analysis of epigenetic regulators revealed high expression levels of several histone-modifying enzymes, such as deacetylases, acetylases, lysine demethylases, and methyltransferases, as well as DNA methylation regulators, including the maintenance methyltransferase DNMT1. Some of these epigenetic regulators showed differential expression across maturation stages, including *SIRT3*, *SIRT6*, *KDM3AP1*, *KMT2E*, *DNMT1* and *DPPA3*. DNMT1 and TET3 were localized in the cytoplasm.

Regarding, the epigenetics modifications 5methylcytosine and 5hmC were observed in the nucleus and in the MII oocyte the 5hmC was also observed on the polar body.

This study provides a comprehensive overview of the transcriptomic dynamics during human oocyte meiotic maturation, offering valuable insights into potential biomarkers for assessing oocyte quality. The distinct transcriptional and epigenetic profiles observed across maturation stages highlight the complexity of oocyte development and suggest potential targets for improving reproductive outcomes.

RESUMO

A maturação meiótica do ovócito humano é um processo crucial para o sucesso da fertilização e do desenvolvimento embrionário. Durante a transição da fase de vesícula germinativa (GV) para a fase de metafase II (MII), ocorrem alterações significativas no núcleo e no citoplasma, permitindo que o ovócito adquira a competência necessária para a fertilização pelo espermatozoide. Essas transformações são influenciadas por mudanças dinâmicas na expressão gênica e na regulação epigenética, que coordenam a ativação, silenciamento e armazenamento de transcritos essenciais.

Compreender a interação entre a regulação transcriptômica e epigenética nos ovócitos é, portanto, crucial para elucidar os determinantes moleculares da qualidade ovocitária. Estes conhecimentos têm implicações significativas na melhoria das tecnologias de reprodução assistida (ART), particularmente na otimização dos protocolos de maturação *in vitro* e na identificação de biomarcadores de viabilidade ovocitária. No entanto, o ovócito humano permanece pouco estudado em comparação com outras espécies de mamíferos, e os mecanismos específicos que sustentam a sua maturação e potencial de desenvolvimento requerem uma investigação mais aprofundada.

No presente estudo, procurámos colmatar estas lacunas de conhecimento, fornecendo uma caracterização detalhada das alterações transcriptômicas dos ovócitos humanos em diferentes fases de maturação (ovócitos GV, MI e MII). Adicionalmente, avaliamos também algumas modificações epigenéticas e reguladores epigenéticos em ovócitos de diferentes fases de maturação.

Para tal, realizámos análises transcriptômicas de ovócitos individuais nas três fases de maturação meiótica e analisámos ovócitos corados com 5-metilcitosina (5mC), 5-hidroximetilcitosina (5hmC), DNMT1 e anticorpos TET3.

A análise transcriptômica de ovócitos revelou um declínio progressivo no número total de genes expressos à medida que os ovócitos amadureciam da fase GV para a fase MII. Especificamente, 9660 genes foram transcritos em ovócitos GV, diminuindo para 8734 na MI e para 5889 na MII. A mesma tendência foi observada para o número de genes expressos exclusivamente em cada fase.

A análise dos genes diferencialmente expressos (DEGs) identificou 1165 DEGs entre os ovócitos GV e MII, com 635 genes com expressão aumentada em MII e 528 com expressão diminuída. Entre GV e MI, foram observados apenas 42 DEGs, dos quais 38 tinham uma expressão aumentada e 4 tinham expressão diminuída em MII, enquanto não foram detectadas alterações significativas na expressão gênica entre MI e MII.

A análise abrangente dos reguladores epigenéticos revelou elevados níveis de expressão de várias enzimas modificadoras de histonas, como desacetilases, acetilases,

desmetilases de lisina e metiltransferases, bem como reguladores da metilação do DNA, incluindo a metiltransferase de manutenção *DNMT1*. Alguns destes reguladores epigenéticos apresentaram expressão diferencial incluindo, *SIRT6*, *KDM3AP1*, *KMT2E*, *DNMT1* e *DPPA3*. *DNMT1* e *TET3* localizaram-se no citoplasma. No que diz respeito às modificações epigenéticas, a 5-metilcitosina e a 5-hidroximetilcitosina foram observadas no núcleo, sendo que, no ovócito MII, a 5hmC também foi detetada no glóbulo polar.

Este estudo fornece uma visão abrangente da dinâmica transcriptômica durante a maturação meiótica do ovócito humano, oferecendo informações valiosas sobre potenciais biomarcadores para a avaliação da qualidade ovocitária. Os diferenciados perfis transcriptômicos e epigenéticos observados entre as fases de maturação destacam a complexidade do desenvolvimento ovocitário e sugerem potenciais alvos para a melhoria dos resultados reprodutivos.

INTRODUCTION

1. Female fertility

The understanding of reproduction among animals, including humans, has advanced significantly since ancient times. The first concept of reproduction, known as "generation," was introduced by Hippocrates in the 5th century BC. He proposed that offspring were produced from "semen" of man and woman, which combined in the uterus to form an embryo (Jones, Withington et al. 1923). Although Aristotle considered that male semen was more important than the matter provided by woman's menstrual blood, later this idea was gradually contradicted over time. In 1651, William Harvey emphasized the importance of "the egg" in reproduction, famously stating, "Omne vivum ex ovo" — "all living things come from eggs." However, it remains unclear what Harvey specifically meant by "egg" (Harvey 1651).

Later, in the late 1600s, Regnier de Graaf discovered that the ovary produced eggs, although he initially believed that the follicle itself was the egg (Graaf 1672). This idea was challenged when Karl Ernst von Baer observed the mammalian oocyte under a microscope and illustrated the oocyte within the ovary follicles in 1827 (von Baer and O'Malley 1956). However, the human oocyte was not studied until the 20th century.

Over the years, discoveries such as the theory of evolution and the existence of DNA have highlighted the complexity and importance of oocyte formation and development. With advances in embryology and genetics, it has become clear that fertility is a delicate process influenced by various factors. In this context, female infertility emerges as a multifactorial issue. According to the World Health Organization (WHO), in 37% of cases of couple infertility, the reason is exclusively female. The most common factors include ovulatory disorders (25%), endometriosis (15%), pelvic adhesions (12%), and tubal blockage (11%), along with other tubal and uterine abnormalities (11%) and hyperprolactinemia (7%).

Thus, the study of reproduction—from the earliest hypotheses of antiquity to contemporary advances—has not only enhanced our understanding of the formation of life but also helped identify the challenges that delay conception, thereby driving research and treatments in the field of reproductive medicine.

2. Folliculogenesis

The ovarian follicles containing the oocytes are the key functional units of female reproduction. The maturation of follicles is a complex process essential not only for supporting oogenesis but also for triggering the production of hormones that are essential for female sexual characteristics and early pregnancy. Follicles are distinguished and classified based on their size, the number of cells, and their morphology. Thus, they can be

classified into the following stages: primordial follicles, primary follicles, secondary follicles and antral follicles (Hertig and Adams 1967).

Follicular development begins with the formation of primordial follicles during fetal development around the fourth month of fetal life. The number of follicles reaches its maximum number around 20 weeks gestation, with around 7 million follicles (Baker 1963). Primordial follicles are characterized by a diameter of around 0.03-0.05mm and contain an oocyte surrounded by a single layer of squamous granulosa cells (GCs). Upon follicle activation, the primordial follicles develop into primary follicles, which are characterized by the change of granulosa cells from squamous to cuboidal shape. In addition, there is an increase in size and the formation of the zona pellucida (ZP), which is a glycoprotein layer that surrounds the oocyte. However, not all primordial follicles initiate growth to develop into primary follicles, some remain quiescent for months or even years (Fortune 2003).

The primary follicles begin to grow as the cuboidal granulosa cells proliferate, forming a multilayer of granulosa cells, which gives rise to the secondary follicles. Besides the multiple layers of GCs, the secondary follicles are also characterised by an outer layer of theca cells (TCs) formed by the condensation of stromal cells (Guahmich, Man et al. 2023).

The follicle progresses to the antral stage, where an antrum cavity develops inside the GC layer. This cavity results from the accumulation of follicular fluid in the GCs. The growth of antral follicles is dependent on gonadotropin and their growth occurs only after puberty (Hennet and Combelles 2012). These lead to the formation of antral follicles with different sizes, between 0.4 and 20 mm, but with similar structures (Sorensen and Wassarman 1976).

Under the influence of luteinizing hormone (LH), the theca wall undergoes significant extracellular matrix remodelling. This, along with the expansion of the antrum, leads to follicular rupture and the release of the oocyte from the selected follicle, known as ovulation (Gougeon 2004).

Antral follicles that reach 2-5 mm are subjected to Follicle-stimulating hormone (FSH) and LH. FSH promotes GC proliferation and the expression of LH receptors in granulosa and theca cells. While LH promotes steroid hormone production and ovulation (Palermo 2007, Casarini, Paradiso et al. 2022). Only one dominant follicle grows to the preovulatory stage (16–29 mm) during each ovarian cycle, while other follicles undergo atresia before reaching 10 mm (Palermo 2007).

Following the dominant follicle's maturation, the LH surge reduces gap junctions in granulosa cells, lowering cyclic guanosine monophosphate (cGMP) levels in the oocyte and resuming meiosis. Then, the preovulatory follicle suffers several changes to facilitate follicle rupture and oocyte release, which include disruption of the granulosa cell basal lamina, and invasion of vascular endothelial cells (Duffy, Ko et al. 2018) After ovulation, the cells within

the follicle undergo transformation, resulting in the formation of the corpus luteum. This structure produces estrogen and progesterone, preparing the endometrium for implantation by creating highly vascularized tissue suitable for supporting a fertilized oocyte. If fertilization occurs, the corpus luteum continues to produce progesterone until approximately 10 weeks of gestation. However, if fertilization does not occur, progesterone levels decrease as the corpus luteum degenerates, leading to the shedding of the endometrium and subsequent menstrual bleeding.

However, in in vitro fertilization (IVF) treatments, the growth of multiple ovarian follicles is stimulated through the administration of supra-physiological doses of FSH. Once the follicles reach an optimal size, a trigger is administered to induce oocyte maturation in preparation for retrieval (Macklon and Fauser 2000).

The complex process of folliculogenesis sets the stage for oogenesis, as the development and maturation of ovarian follicles provide the structural and hormonal environment necessary for the growth and meiotic progression of the oocyte.

3. Oogenesis

The female gamete, the oocyte, is one of the largest cells in humans with a diameter of approximately 100µm when mature and has the function to give half the chromosomal complement to the embryo. Although the spermatozoon also makes a genetic contribution to the zygote, the oocyte is its major cytoplasmic donor, supplying organelles and molecules essential for development. Therefore, the oocyte can initiate and sustain the development of a complete organism (Conti and Franciosi 2018).

Oocytes are unique and complex due to their ability to form totipotent cells, giving rise to every cell type in the adult organism, but at the same time being highly specialised cells. This highly specialized cell is derived from primordial germ cells (PGCs) isolated during embryonic development and first detected in the wall of the yolk sac near the allantois from 3-4 weeks after fertilization (De Felici 2004). Then, PGCs initiate migration to the gonadal ridge and start to divide and proliferate (mitosis) which occurs mainly after their arrival to the ridge. Until the sixth week after fertilization, both the PGC and gonads remain bipotential and the sex determination of the ovary and testis occurs after week 7 after fertilization (Mamsen, Brchner et al. 2012). Once PGC arrives at the developing gonad, they stop migrating but continue dividing. At this point, these diploid cells are known as oogonia and continue dividing until they receive signals from surrounding somatic cells to stop mitosis and start meiosis.

Following the last mitotic division, around 11-12 weeks of gestation, each oogonium divides and enters the initial stage of meiosis (meiosis I) to become the primary oocyte. Not all

oogonia progress to the first meiotic division, the number of germ cells declines sharply and most oogonia become atretic during this period. The peak in germ cell numbers occurs at 20 weeks of gestation, following a remarkable decline in germ cell numbers (Findlay, Hutt et al. 2015).

The surviving oogonia progresses to meiosis, in which cells replicate DNA before two cell divisions occur- Meiosis I and Meiosis II. The primary oocyte is formed after oogonia initiates the first meiotic division, when the cell is arrested at prophase I.

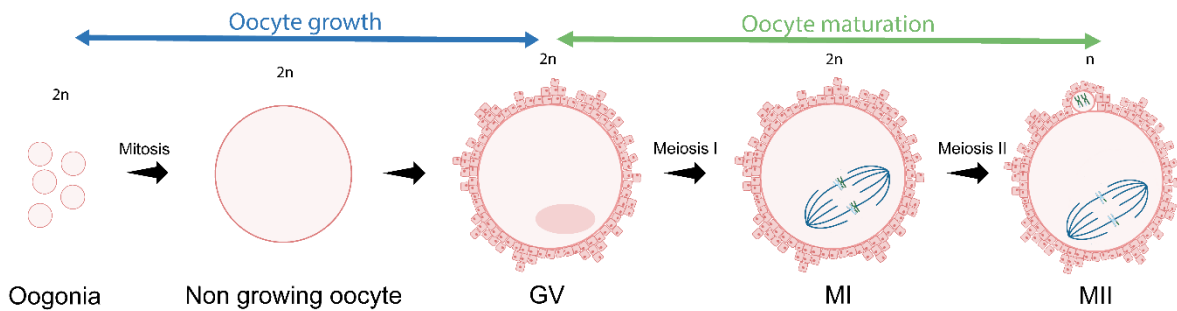


Figure 1 Oogenesis. The process begins with the multiplication phase, where oogonia undergo mitotic divisions. This is followed by the growth phase, during which the oogonia develop into primary oocytes. In the maturation phase, meiosis occurs, producing a secondary oocyte and polar bodies. *Meiosis II* is only completed upon fertilization, resulting in the formation of a mature oocyte. GV: Germinal vesicle, MI: Metaphase I, MII: Metaphase II.

3.1. Meiosis I-Prophase I

Prophase I has five phases: leptotene, zygotene, pachytene, diplotene and diakinesis. The oogonia progress through several subphases until it is arrested in the diplotene, for several years in humans (Wang and Pepling 2021). This subphase is the most stable for oocytes due to the chromosome's conformation, where homologous chromosomes (maternal and paternal) are connected at chiasmata (where crossing over occurs during recombination). During leptotene, the first substage of prophase I, the homologous chromosomes begin to align, and the double-strand break (DSB) is formed. Additionally, early synaptonemal complex (SC) assembly occurs, where the homologs are aligned with each other by a structure of proteins and that is required to stabilise homologous pairing interactions and promote interhomolog crossover formation. The formation of this complex is dependent of SYCP2 (Synaptonemal complex protein 2) and SYCP3 (Synaptonemal complex protein 3), which are initially axial proteins (assemble onto meiotic chromosome axes) that become lateral proteins when the complex is assembled. The SC also consists of central proteins (located in the centre of the SC), such as SYCE1 (Synaptonemal Complex Central Element Protein 1), SYCE2 (Synaptonemal Complex Central Element Protein 2), SYCE3 (Synaptonemal Complex Central Element Protein 3), and TEX12 (Testis Expressed 12),

and transverse proteins (bridge the parallel homologous axes), such as SYCP1 (Gao and Colaiácovo 2018). The importance of these proteins and SC assembly is observed when alterations in genes encoding these proteins are related to miscarriages, infertility and birth defects in humans and mouse (de Vries, Behar et al. 2014, Sánchez-Sáez, Gómez-H et al. 2020).

The SC complex is not fully assembled during leptotene stage. The completion of the synaptonemal complex occurs specifically during the pachytene phase, marked by the assembly of nearly all lengthwise-aligned homologous chromosome pairs.

The exchange of genetic material between homologous chromosomes is made possible by the DSBs, created by SPO11 topoisomerase during the leptotene stage (Keeney 2008), and is essential for fertility and genetic diversity. The indispensable role of this topoisomerase was demonstrated in mouse oocytes when Spo11 mutant females showed defects in follicle formation due to the absence of DSB formation (Di Giacomo, Barchi et al. 2005). This enzyme does not act alone; its catalytic activity is influenced by auxiliary proteins such as REC114 (REC114 Meiotic Recombination Protein), MEI4 (Meiotic Double-Stranded Break Formation Protein 4), and IHO1 (Interactor Of HORMAD1 1) (Kumar, Oliver et al. 2018).

In addition to DSB generation, DSB repair has also been shown to be essential for proper oocyte/ovary development. This mechanism begins with end processing, which involves cleavage by an endonuclease. This endonuclease cleaves small fragments that are associated with SPO11. Subsequently, the single strand of DNA is extended by exonucleases and the homologous strand is invaded to repair the DSB through the activity of the recombinases RAD51 (RAD51 Recombinase) and DMC1 (DNA Meiotic Recombinase 1), forming a displacement (or D) loop in the homolog which is subsequently extended by the action of the helicase HFM1 (Helicase For Meiosis 1).

Two mismatch repair proteins, MSH4 (MutS Homolog 4) and MSH5 (MutS Homolog 5), stabilize a double Holliday junction intermediate when a second end capture occurs, promoting crossovers. Later, RNF212 (Ring Finger Protein 212), HEI10 (E3 ubiquitin-protein ligase CCNB1IP1), and CNTD1 (Cyclin N-terminal domain-containing protein 1) play a regulation role in the crossover selection and the resolution of final products is promoted by MLH1 (MutL Homolog 1), MLH3 (MutL Homolog 3), and EXO1 (Exonuclease 1) (Holloway, Sun et al. 2014, Cannavo, Sanchez et al. 2020, Wang and Pepling 2021, Gioia, Payero et al. 2023). The resolution of recombination occurs in the diplotene phase, following the initiation of the SC disassembly, the resolution of the D-loop and the repair of DSBs. Therefore, at the end of the diplotene phase, where the oocyte remains arrested for years (in humans), the homologous chromosomes are connected at the sites of recombination (chiasmata). The completion of prophase I in oocytes occurs with the transition from the prolonged diplotene arrest to diakinesis, marked by chromosome condensation, chiasmata

terminalization, nuclear envelope breakdown, and spindle assembly, preparing the oocyte for metaphase I upon hormonal stimulation.

3.2. Oocyte maturation

Oocyte maturation consists of closely linked processes, namely nuclear and cytoplasmic maturation (Eppig, O'Brien et al. 1996). Both are indispensable for producing an egg capable of fertilization and subsequent development into viable offspring. Among these, nuclear maturation involves the changes within the oocyte necessary for its progression from an immature to a mature state, with emphasis on alterations in the nuclear components (Swain and Smith 2006). Cytoplasmic maturation involves numerous essential changes that prepare the oocyte for activation, fertilization, and preimplantation development (Eppig, O'Brien et al. 1996).

3.2.1. Nuclear maturation

Oocyte maturation is the final stage of oogenesis, during which a diploid oocyte undergoes meiotic division to produce a haploid cell. As previously mentioned, during embryonic development, mammalian oocytes become arrested at the diplotene stage of prophase I of meiosis, retaining a germinal vesicle (GV) - a prominent nucleus enclosed by a nuclear envelope (De La Fuente 2006). The maintenance of oocyte meiotic arrest relies on molecules such as cyclic adenosine monophosphate (cAMP) within the growing oocytes (Cho, Stern et al. 1974, Conti, Andersen et al. 2002), as well as the NPPC (natriuretic peptide precursor type C)/NPR2 (natriuretic peptide receptor 2) system in the somatic GC surrounding the oocyte. One of the key factors in oocyte maturation is cAMP, as high cAMP levels within the oocyte lead to meiosis arrest, while a reduction in cAMP triggers the resumption of meiosis (Conti, Andersen et al. 2002, Zhang, Ouyang et al. 2009). cAMP levels are regulated by adenylyl cyclase (AC), which catalyzes adenosine triphosphate (ATP) to form cAMP, and by phosphodiesterases, which break down cAMP. AC activity depends on G protein-coupled receptor 3 (GPR3) and GS protein, both of which are required to maintain meiotic arrest (Hinckley, Vaccari et al. 2005, Mehlmann 2005, Diluigi, Weitzman et al. 2008). Inhibition of any of these proteins disrupts the ability of the oocyte to sustain meiotic arrest.

Additionally, oocytes coordinate with the surrounding granulosa cells to maintain high cAMP levels, thereby preventing the resumption of meiosis. Therefore, sustaining high cAMP levels within the oocyte relies on cGMP produced from guanosine triphosphate (GTP) by guanylyl cyclases in the surrounding granulosa cells. Briefly, natriuretic peptide precursor C (NPPC) in mural granulosa cells binds to natriuretic peptide receptor 2 (NPR2) on cumulus

cells, producing cyclic guanosine monophosphate (cGMP). cGMP enters oocytes through gap junctions and inhibits phosphodiesterase PDE3A, maintaining high cAMP levels (Zhang, Su et al. 2010, Casalechi, Dias et al. 2019). Elevated cAMP inhibits maturation-promoting factor (MPF) activity via a protein kinase A (PKA)-dependent mechanism. MPF, composed of CDK1(Cyclin-dependent kinase 1) and cyclin B, is regulated by phosphorylation at specific sites (tyrosine 15 and threonine 14) by the kinase WEE1B (WEE2 Oocyte Meiosis Inhibiting Kinase) and dephosphorylation by the phosphatase CDC25(Cell Division Cycle 25). The CDK1 and cyclin B complex, namely MPF, is essential for oocyte maturation (Adhikari, Busayavalasa et al. 2016, Jaffe and Egbert 2017).

Following the LH surge from the pituitary, changes in cAMP levels also occur that promote the resumption of meiosis in the GV-stage oocyte (Lindner, Tsafiriri et al. 1974). LH binds to its receptor (LHR), triggering a decrease in cGMP levels within granulosa cells by downregulating the NPPC/NPR2 signalling pathway and limiting cGMP diffusion into oocytes (Zhang, Su et al. 2010). Additionally, gap junctions between oocyte and granulosa cells are inactivated after LH binding, reducing intra-oocyte cAMP levels.

A decrease in cAMP levels within oocytes activates the MPF, leading to chromosome segregation and germinal vesicle breakdown (GVBD)(Schorderet-Slatkine, Schorderet et al. 1978). The nuclear envelope destabilisation occurs due to lamin phosphorylation by CDK1 (Adhikari, Busayavalasa et al. 2016).

The mitogen-activated protein kinase (MAPK) cascade, along with MPF, plays a role in meiotic cell cycle progression in oocytes. MAPK increases MPF stability by suppressing inhibitors and activating CDC25 phosphatase. MAPK is not essential for the initiation of meiosis but is crucial for the post-GVBD event (Wang, He et al. 2007).

The disintegration of the nuclear envelope, followed by the assembly of microtubules in the nuclear region and their attachment to chromosome kinetochores allows the formation of a bipolar spindle, which leads to the alignment of the chromosomes along the spindle equator, resulting in the transient metaphase I (MI) stage. Immediately afterward, the spindle migrates to the oocyte surface and segregates half the homologous chromosomes into a small cell, resulting in an asymmetric division and forming the polar body.

After completing the first meiotic division, a second meiotic spindle captures the chromosomes and organizes them on the equator, which represents the metaphase II (MII) stage. Oocytes remain arrested at the MII stage until fertilization occurs (Clift and Schuh 2013). This transition, triggered by hormonal surge, takes around 34 hours to complete and leads to ovulation. However, studies show that the time interval may vary among individuals, ranging from 22 to 56 hours (Erden, Mumusoglu et al. 2022). Therefore, the oocyte arrested at metaphase II is ovulated into the female reproductive tract, in the fallopian tube, and meiosis II is only completed if fertilization occurs, when sperm enters the oocyte. This

second meiotic division is also asymmetric and gives rise to a one-cell embryo and the second polar body, allowing early embryonic development (Clift and Schuh 2013).

3.2.2. Cytoplasm maturation

Cytoplasmic maturation involves the expression and degradation of maternal transcripts and the reorganization of organelles, such as mitochondria endoplasmic reticulum (ER), cortical granules, and ribosomes. This process is essential for the oocyte to acquire the competence required for successful fertilization and subsequent embryonic development. Studies using transmission electron microscopy have shown that oocyte maturation from the GV stage to MII is characterized by cytoplasmic remodelling. These key changes include the redistribution of organelles, disassembly of the Golgi apparatus (GA), accumulation of cortical granules, and the formation of mitochondria–endoplasmic reticulum complexes (Trebichalská, Kyjovská et al. 2020).

Organelles play an important role in oocyte maturation and embryogenesis since they reorganize and store the mRNA, proteins and transcription factors required for these developmental stages. Therefore, changes in localization, morphology, and biochemical properties of organelles influence oocyte competence (Mao, Lou et al. 2014).

The mitochondria are one of the main cytoplasmic organelles, which play a fundamental role in energy production and calcium homeostasis, essential for successful fertilization and early embryonic development. The mitochondria are the only organelles with their own DNA, the mitochondrial DNA (mtDNA), similar to the nucleus (Anderson, Bankier et al. 1981). Mitochondria redistribute throughout the oocyte cytoplasm, often clustering in areas of high energy demand. Therefore, the mitochondria distribution and the mtDNA copy number alter significantly during oocyte maturation (Hao, Zhao et al. 2024). Primordial germ cells contain a small amount of mtDNA. However, mtDNA copies increase significantly, reaching their peak at the MII stage. Two critical phases of rapid mtDNA amplification are observed: first, during the transition from the primordial to the primary follicle stage, and second, during the transition from GV to MII oocytes at the late antral follicle stage (Van Blerkom 2004, Kirillova, Smits et al. 2021). Abnormal mitochondrial rearrangement and low ATP levels were associated with decreased oocyte quality (Zhang, Wu et al. 2006).

The ER plays a pivotal role in calcium signalling, being the major intracellular Ca^{2+} store. In oocytes, it manages the storage and release of Ca^{2+} into the cytoplasm via Ca^{2+} -ATPases, Ca^{2+} storage proteins, and Ca^{2+} release channels, which are essential for maintaining intracellular Ca^{2+} homeostasis (Mehlmann, Terasaki et al. 1995). The release of Ca^{2+} is mediated through the inositol 1,4,5-trisphosphate (IP3) receptor or the ryanodine receptor, both of which are located in ER membrane (Machaca 2004). Studies demonstrate that the affinity of the IP3 receptor increases during oocyte maturation, which releases Ca^{2+} into the

cytoplasm and increases Ca^{2+} concentration (Machaca 2004, Wakai and Fissore 2013). However, this concentration is also dependent on Ca^{2+} influx from the extracellular environment, in which STIM1 (Stromal Interaction Molecule 1) plays a role. STIM1, a transmembrane-spanning Ca^{2+} -binding protein, has a Ca^{2+} sensor domain localized in the luminal compartment of the ER (Koh, Lee et al. 2009). The ER's role extends to facilitating Ca^{2+} oscillations following sperm fertilization, which is critical for completing the second meiotic division, recruiting maternal mRNAs, and preventing polyspermy (Ajduk, Ciemerych et al. 2008). This functionality depends on the proper reorganization of the ER during oocyte maturation (Mehlmann, Terasaki et al. 1995, FitzHarris, Marangos et al. 2007). In human MII oocytes, ER clusters are distributed throughout the oocyte. The smooth ER (SER) exists as isolated vesicles or peripheral aggregates, which increase during preovulatory maturation. In contrast, the rough ER (RER) is absent in mature oocytes (Mao, Lou et al. 2014).

The Golgi apparatus is distributed throughout the cytoplasm in the GV oocytes; however, GA fragments after GVBD and is no longer essential for oocyte maturation (Moreno, Schatten et al. 2002). The GA undergoes progressive disintegration during maturation, occasionally detected in MI oocytes but not in MII oocytes (Trebichalská, Kyjovská et al. 2020). In addition, GA generates cortical granules (CGs) that are found in the cortex of unfertilized oocytes and play important roles in fertilization, helping to prevent polyspermy by modifying the ZP (Sathananthan, Ng et al. 1985, Cheeseman, Boulanger et al. 2016). In human oocytes, CGs are abundant under the oolemma in MI and MII oocytes but sparse in GV oocytes (Trebichalská, Kyjovská et al. 2020).

The degradation and translation of maternal transcripts are essential for cytoplasmic maturation to be accomplished. Maturing oocytes, which lack active transcription, depend entirely on maternal mRNAs that are stored during earlier stages. The GV oocyte contains numerous maternal transcripts that remain translationally dormant until meiosis resumes. These transcripts encode proteins essential for processes such as spindle assembly, the maintenance of metaphase II arrest, and mRNA clearance (Sha, Dai et al. 2017). The stability and translational activation of these mRNAs within the oocyte are regulated through cytoplasmic polyadenylation at the 3'-untranslated region (3'-UTR). This process, mediated by the cytoplasmic polyadenylation element (CPE) and CPE-binding proteins, involves the addition of multiple adenosine monophosphate (poly(A)) tails to the mRNA (Piqué, López et al. 2008). This temporal regulation of maternal transcripts, stored in the cytoplasm, is important for competency acquisition and embryogenesis. Conversely, deadenylation and loop formation between mRNA termini repress mRNA translation (Reyes and Ross 2016).

4. Cumulus cells and oocyte interaction

GCs are somatic cells that provide the physical support and microenvironment essential for oocyte growth and maturation (Huang and Wells 2010). Initially, these cells appear in the primordial follicle with a squamous appearance. As folliculogenesis progresses, they differentiate into a cuboidal form. In the luteal phase, they transform into hypertrophied lutein cells. The differentiation and proliferation of GCs provide essential support for oocyte growth, a process regulated by various hormones and growth factors (Tapanainen, Leinonen et al. 1987, Robker and Richards 1998). Granulosa cells differentiation begins when the follicular antrum is formed. This process generates two types of granulosa cells, the mural granulosa cells (MGCs) and cumulus cells (CCs). MGCs surround the antrum and have the greatest steroidogenic activity, while CCs enclose the oocyte. The proximity of cumulus cells to the oocyte facilitates the formation of the cumulus-oocyte complex (COC), enabling communication through gap junctions (Buccione, Vanderhyden et al. 1990, Gilchrist, Lane et al. 2008).

The communication occurs via gap junctions and paracrine/juxtacrine signalling factors, which enable cumulus cells to penetrate the ZP and establish connections with the oocyte cytoplasm (Gilula, Epstein et al. 1978). This communication is achieved through the formation of transzonal projections (TZPs), cytoplasmic projections that penetrate the ZP. Thus, the TZPs of the CCs through the ZP enable the supply of factors and regulatory molecules to oocytes (Baena and Terasaki 2019). This enables critical bidirectional communication between cumulus cells and oocytes. Cumulus cells supply metabolic products like pyruvate, cholesterol, and amino acids that oocytes cannot produce or uptake independently (Leese and Barton 1985, Haghighat and Van Winkle 1990, Su, Sugimura et al. 2008). They also support oocyte energy metabolism, transcriptional regulation, and meiotic control (De La Fuente and Eppig 2001, Norris, Ratzan et al. 2009). On the other hand, oocytes regulate cumulus cell functions by secreting growth factors that promote proliferation and prevent apoptosis. For example, growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) are oocyte-secreted factors that enable CC expansion during oocyte maturation (Su, Wu et al. 2004, Mottershead, Sugimura et al. 2015), ensuring optimal oocyte quality. Oocytes also influence cumulus cell metabolic activities, such as glycolysis and cholesterol biosynthesis, and regulate cGMP production critical for meiotic arrest (Zhang, Su et al. 2010).

This dynamic between cumulus cells and the oocyte not only supports the processes of oocyte growth and maturation but also governs critical mechanisms such as meiotic arrest, resumption, and oocyte quality, bridging the roles of cellular communication and developmental competence.

As mentioned before, the GV oocyte initially remains arrested at prophase I due to elevated levels of cAMP within the oocyte. Granulosa cells produce cAMP, which is transferred to the oocyte through gap junctions. Although the oocyte also generates cAMP, its meiotic arrest is further maintained by cGMP produced by GCs and transferred via gap junctions (Cho, Stern et al. 1974, He, Zhang et al. 2021).

In contrast, meiotic resumption is triggered by a reduction in intra-oocyte cAMP levels. LH stimulates cAMP production and activates the MAPK pathway. MAPK activity disrupts gap junction communication, and cyclic nucleotide levels (cGMP and cAMP) decrease in the oocyte, thereby inducing meiotic progression (Wang, He et al. 2007).

In addition to their role in meiotic control, cumulus cells also play a critical role in fertilization by assisting sperm penetration and binding to the ZP (Chung, Chiu et al. 2009).

Studies evaluating CC apoptosis and genetic content have established a negative correlation between CC apoptosis and oocyte quality (Lee, Joo et al. 2001, Lemseffer, Terret et al. 2022), reinforcing the importance of CC and oocyte interaction for oocyte development.

5. Gene expression regulation during oocyte maturation

Oocyte maturation is a complex process involving folliculogenesis and oogenesis, which are characterized by distinct transcriptional states and transcriptomic transitions. Oocyte development begins during fetal development when oogonia enter meiosis and become arrested at prophase I. After puberty, a surge in LH resumes maturation, leading to meiotic divisions until MII. This process involves transcriptional, organelle, and epigenetic changes, essential for fertilization and early embryonic development.

During growth, oocytes remain transcriptionally active while accumulate maternal mRNAs. These mRNAs are stored in message ribonucleoprotein complexes and in the cytoplasm and then translated when required (Hafidh, Capková et al. 2011). However, transcription ceases before oocyte maturation, specifically at the prophase-arrested GV stage. At this point, chromatin undergoes significant condensation and surrounds the nucleolus, a configuration that marks the transition to transcriptional silence (De La Fuente, Viveiros et al. 2004, Clift and Schuh 2013). Transcription is reactivated only after fertilization, during the early cleavage embryo. Traditionally, embryonic genome activation (EGA) was believed to begin between the four- and eight-cell stages in human preimplantation development (Braude, Bolton et al. 1988). However, recent findings indicate that EGA may start as early as the one-cell stage (Asami, Lam et al. 2022). During transcriptional quiescence, post-transcriptional regulation ensures the storage and timely activation of maternal factors essential for the oocyte-to-embryo transition development (Clarke 2012).

The transcripts stored to support oocyte maturation are selectively degraded during the maturation process to ensure the transition from maternal to embryonic control of gene expression (Su, Sugiura et al. 2007, Clift and Schuh 2013). The maternal mRNAs, which are stable and remain in the oocyte until ovulation, suffer a change in their stability and are marked to be degraded (Paynton, Rempel et al. 1988). The degradation of these transcripts starts once oocyte maturation begins and extends beyond fertilization. During oocyte maturation, the transcripts responsible for sustaining metaphase II arrest remain intact, while those involved in prophase I arrest are degraded (Su, Sugiura et al. 2007).

These maternal mRNAs translation and degradation processes are regulated through post-transcriptional modification such as polyadenylation and deadenylation, since transcription is postulated to be absent during oocyte maturation (Paynton, Rempel et al. 1988).

Maternal mRNAs undergo polyA tail lengths modifications during oocyte maturation. Early studies in *Echinoidea* eggs revealed that the mRNA of unfertilized eggs had short or no polyA tails, whereas fertilized eggs and embryos contained mRNAs with long polyA tails (Slater, Slater et al. 1972). Later, it was also demonstrated that immature oocytes contain transcripts with shorter poly-adenylated tails (Clift and Schuh 2013). The translation of these maternal transcripts is strongly correlated with the length of polyA tails. Following the onset of oocyte maturation, polyadenylation of dormant transcripts occurs in the cytoplasm, activating these mRNAs and enabling them to be translated (Clift and Schuh 2013, Liu, Zhao et al. 2023).

The removal of adenosines from the mRNA poly(A) tail, a process called deadenylation, occurs in the maternal transcripts during oogenesis and early embryogenesis to prepare the oocyte for future quiescence. However, mRNAs can avoid deadenylation if they contain specific motifs in their 3' UTR, such as a polyadenylation signal (PAS) and a CPE. PAS, typically AAUAAA or AUUAAA, is recognized by the Cleavage and Polyadenylation Specificity Factor (CPSF). In contrast, the CPE, a U-rich sequence, is recognized by CPEB. CPEB-mediated polyadenylation can be influenced by factors like the number of CPEs and PAS, as well as the distance between them. CPEB, depending on its phosphorylation state, can recruit either PARN to promote deadenylation or the polyA polymerase GLD2 (Terminal Nucleotidyltransferase 2) to facilitate polyadenylation of CPE-containing mRNAs (Fig.2). After cleavage, the poly(A) tail is added by a nuclear poly(A) polymerase (Lorenzo-Orts and Pauli 2024). Poly(A) tail promotes translational efficiency and protects mRNA from degradation in the cytoplasm. During oogenesis and early embryogenesis, the regulation of maternal mRNA poly(A) tail length regulates translation. In general, this process involves the 5' cap-binding protein eIF4E (Eukaryotic Translation Initiation Factor 4E) and the 3' poly(A)-binding protein PABPC (Poly(A) Binding Protein Cytoplasmic). In oocyte, the levels of PABPC are limited but in somatic cells are abundant and support translation of mRNAs

with short poly(A) tails. Therefore, PABPC preferentially binds to mRNAs with long poly(A) tails, enhancing their translation. Overexpressing of PABPC in oocytes enhances translation of mRNAs with short poly(A) tails. Deadenylated maternal mRNAs can be stabilized in RNA granules by RNA-binding proteins, such as eIF4E1b (Eukaryotic Translation Initiation Factor 4E Family Member 1B) in vertebrates. However, in somatic cells, short poly(A) tails are decapped and degraded. Thus, the limited availability of PABPC helps coordinate poly(A) tail length with translation efficiency (Xiang and Bartel 2021, Lorenzo-Orts and Pauli 2024).

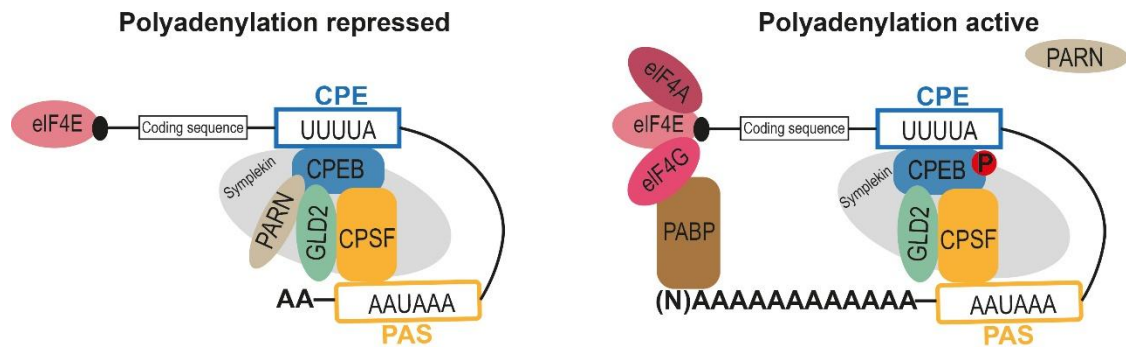


Figure 2 CEB-mediated translational control. In oocytes, messenger RNAs containing the cytoplasmic polyadenylation element (CPE) remain translationally repressed within a complex that includes the non-phosphorylated CPE-binding protein (CPEB) and PARN, an enzyme that promotes deadenylation. Upon CPEB phosphorylation, PARN is removed, leading to the activation of the GLD2 poly(A) polymerase and subsequent polyadenylation. Adapted from (Radford, Meijer et al. 2008).

In addition to these interactions, there are also RNA modifications, namely 5-methylcytosine (m^5C) and N6-methyladenosine (m^6A), which are involved in the regulation of maternal mRNA stability (Lorenzo-Orts and Pauli 2024). These are epigenetic marks in which a methyl group is added to the fifth and sixth position of a cytosine and adenosine in RNA, respectively. These and other epigenetic modifications will be discussed in more detail below.

6. Epigenetics of Germ Cells Development

The development of a fertilized zygote into a complex organism is driven by epigenetic regulation, which plays a crucial role in growth, development, and cell differentiation. Epigenetics refers to the study of meiotically and/or mitotically heritable changes in gene function and expression that are not caused by changes in the DNA sequence itself, but rather by chemical modifications in the chromatin. These modifications can either enhance or repress transcription by altering chromatin structure and the binding of transcription factors (Russo, Martienssen et al. 1996, Felsenfeld 2014).

Crucial epigenetic modifications include DNA methylation and hydroxymethylation, histone modifications, and RNA modifications. Additionally, some researchers consider non-coding

RNAs as epigenetic regulators. Epigenetic regulation is carried out by specialized enzymes categorized as "writers," "readers," and "erasers" of these chemical marks (Peixoto, Cartron et al. 2020).

Epigenetic regulation plays a pivotal role in the complex process of oogenesis, ensuring the proper development and function of female gametes. The primordial germ cells are the precursors to gametes; therefore, a “clean slate” is necessary to establish gamete-specific epigenomes. Indeed, this reset is achieved through two major global reprogramming events in mammals. The first reprogramming event occurs in PGCs, whereby genome demethylation occurs upon entering the genital ridge. During that period, the imprinting marks on the maternal and paternal chromosomes are erased, followed by *de novo* methylation to establish the new sex-specific imprints during gametogenesis. The second reprogramming event takes place after fertilization. Following the fusion of the gametes, global demethylation occurs, except for imprinting marks that are maintained throughout adulthood. In the paternal pronucleus, demethylation is actively facilitated by TET enzymes and is completed before the first DNA replication. Conversely, the maternal methylome is passively removed during subsequent cleavage divisions, since the oocyte-specific DNMT1 is mostly excluded from the nucleus (Ishida and Moore 2013, Marshall and Rivera 2018). After birth, the epigenome of female germ cells is progressively established during oogenesis, while in male germ cells, DNA methylation patterns are completed before birth (Kurimoto and Saitou 2019). Alongside DNA methylation, histone modifications also play a crucial role in germ cell development. These modifications are highly dynamic throughout oogenesis and are essential for proper regulation. Curiously, maternal pronuclear histones are hypermethylated, holding both repressive and active marks (Marshall and Rivera 2018, Chamani and Keefe 2019).

In the following subsections, each type of epigenetic modification—DNA methylation and hydroxymethylation, histone modifications and RNA modifications—will be discussed in detail to highlight their specific contributions to oocyte maturation.

6.1. DNA methylation and DNA hydroxymethylation in oocyte

DNA methylation is one of the first epigenetic modifications described. This modification is catalyzed by the DNA methyltransferase (DNMT) protein family, which is responsible for adding a methyl group (-CH₃) to the fifth carbon of cytosine bases, predominantly in CpG dinucleotides, yielding a 5-methylcytosine (5mC) (Jones and Takai 2001, Portela and Esteller 2010). These CpG dinucleotides are found in high concentrations around promoter regions, which when methylated generally repress gene transcription. The known DNMTs have slightly different functions, with only DNMT1, DNMT3A and DNMT3B having

methyltransferase activity. DNMT1 maintains methylation patterns during cell division with semi-conservative DNA replication, while DNMT3A and DNMT3B are responsible for de novo methylation, which methylates unmethylated DNA. DNMT3L, is a non-canonical family member; it lacks catalytic DNMT activity but facilitates DNMT3A and DNMT3B activity (Portela and Esteller 2010, Lyko 2018, Peixoto, Cartron et al. 2020). Among these, DNMT2 has a distinct function in transfer RNA methylation (Goll, Kirpekar et al. 2006). For decades, 5mC has been a major focus of epigenetic research. However, another epigenetic mark that was later revealed was 5hmC, which results from 5mC oxidation by the action of the ten-eleven translocation (TET) protein family, namely, TET1, TET2, and TET3. Subsequently, TET-mediated oxidation of 5hmC can occur to originate 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Tahiliani, Koh et al. 2009, Branco, Ficz et al. 2011). DNA hydroxymethylation supports the role of 5hmC in active demethylation, which occurs through the action of thymine-DNA glycosylase followed by the base excision repair pathway by alternative mechanisms (Maiti and Drohat 2011).

As mentioned before, oocytes experience extensive epigenetic remodelling, characterized by global DNA demethylation in primordial germ cells and the subsequent establishment of an oocyte-specific DNA methylome during oogenesis, which begins in the primary oocyte (Reik, Dean et al. 2001). Although DNA methylation dynamics play a crucial role in oocyte growth and maturation, the specific mechanisms and patterns of DNA methylation in human oocytes during maturation remain poorly understood and are still under investigation.

The oocyte has a unique DNA methylation pattern, distinct from sperm or somatic cells. Mature oocytes show around 40% global DNA methylation, unlike sperm, which have around 90%. Subsequently, there are several germline differentially methylated domains (gDMRs) (Smallwood, Tomizawa et al. 2011, Kobayashi, Sakurai et al. 2012, Veselovska, Smallwood et al. 2015). In human oocytes, the percentage of global DNA methylation is relatively higher, around 48% to 53.8% (Guo, Zhu et al. 2014, Okae, Chiba et al. 2014, Yu, Dong et al. 2017, Ye, Yang et al. 2020).

DNA methylation in oocytes is acquired gradually during growth and development. In primordial follicles, non-growing oocytes (NGOs) are largely unmethylated. As oocytes grow and follicles develop, methylation increases, particularly during the later stages of folliculogenesis (Smallwood, Tomizawa et al. 2011).

DNMTs play a crucial role in establishing DNA methylation during oocyte development, though patterns differ from somatic cells. DNMT1 is localized in the cytoplasm and DNMT3A/3B in the nucleus of GV and MII oocytes, with DNMT1 and DNMT3B increasing in MII (Lucifero, La Salle et al. 2007, Uysal, Ozturk et al. 2017). In mouse oocytes, DNA methylome requires the Dnmt3a and its co-factor Dnmt3l (Bourc'his, Xu et al. 2001, Kaneda, Okano et al. 2004). In humans, DNMT1 and DNMT3A are expressed in all maturation stages

of oocytes, while DNMT3B is not expressed in non-growth oocyte (Okada, Chiba et al. 2014, Yan, Gu et al. 2021). DNMT1 is localized in the nucleus in GV oocytes and in the cytoplasm in MI and MII oocytes, suggesting its role in methylation maintenance. DNMT3A and DNMT3B are found in the cytoplasm of all stages but not in the nucleus (Petrussa, Van de Velde et al. 2014).

Studies on DNA methylation and its epigenetic regulators (DNMTs) in oocytes have been conducted in humans and mice. However, research on DNA hydroxymethylation and TET enzymes during oocyte maturation remains limited.

In human oocytes, the presence of 5hmC was observed, and a slight decrease in its signal was observed during maturation (Petrussa, Van de Velde et al. 2014, De Munck, Petrussa et al. 2015). In fact, it was observed that 5hmC constituted 1.60% of the genome with higher 5hmC levels in CpG islands (CGIs) compared to gene bodies, promoters, enhancers, and repetitive elements (Yan, Gu et al. 2021). Additionally, the deficiency of TETs, the enzymes responsible for converting 5mC into 5hmC, was associated with an embryonic arrest at the 2-cell stage and affected global DNA methylation (Arand, Chiang et al. 2022). Additionally, Tet3 exhibits high expression levels in MII oocytes, suggesting an important role in DNA reprogramming in zygotes (Gu, Guo et al. 2011, Wossidlo, Nakamura et al. 2011, Qi, Wang et al. 2022).

6.2. Histone modifications in oocyte maturation

Histone modifications play a critical role in epigenetic regulation by influencing chromatin structure and remodelling. The nucleosome, the fundamental repeating unit of chromatin, consists of an octameric histone protein complex, including the linker and stabilizer histone, H1, along with pairs of core histones (H2A, H2B, H3, and H4). While multiple variants of histones H2A, H3, and H1 have been well characterized, variants of H2B and H4 remain less studied (Joseph and Young 2023). Histone modifications come in various forms, including acetylation, methylation (mono-, di-, or tri-methylation), phosphorylation, ubiquitination, and sumoylation. Histone acetylation is catalysed by histone acetyltransferase enzymes (HATs), whereas histone deacetylases (HDACs) mediate deacetylation. These enzymes are not specific to a single residue and promote a transcriptionally permissive chromatin structure. In contrast, histone methylation, can either activate or repress transcription depending on the residue modified and the number of methyl groups added. This process is catalyzed by histone methyltransferases (HMTs), while lysine demethylases (KDMs) facilitate demethylation. Another key modification is histone phosphorylation, which occurs on serine, threonine, and tyrosine residues. This process, mediated by various regulatory kinases, alters the affinity between DNA and histones, thereby affecting transcriptional regulation. Additionally, histone ubiquitination and

sumoylation are important post-translational modifications. Both processes involve a cascade of three enzymes: E1 (activating enzyme), E2 (conjugating enzyme), and E3 (ligase enzyme). Ubiquitination can either promote or suppress transcription depending on the specific site of modification. In contrast, sumoylation, which involves the attachment of small ubiquitin-like modifiers (SUMO) to histones or transcription factors, typically acts as a repressive mark for gene transcription (Chamani and Keefe 2019, Peixoto, Cartron et al. 2020).

In contrast to DNA methylation and DNA hydroxymethylation, histone modifications in human oocytes have been studied more extensively and their function and regulation in oocyte and embryonic development is more established (Gu, Wang et al. 2010). The oocyte undergoes multiple posttranscriptional modifications, such as acetylation, phosphorylation, methylation, glycosylation, ubiquitination and sumoylation.

The histone acetylation is controlled by HATs and HDACs (He, Zhang et al. 2021). HDACs are important in oocyte development and maturation. HDAC1 and HDAC2 are localized in the nucleus throughout oocyte growth. HDAC1 decreases progressively during growth and HDAC2 increases post-partum in the nucleus and remains stable throughout oocyte growth (Ma, Pan et al. 2012). HDAC3 plays a crucial role in spindle organization and chromosome alignment during meiosis. In GV oocytes, HDAC3 is localized in the nucleus and disperses after GVBD, accumulating on the meiotic spindle until MII. Additionally, this histone acetylase also plays an important role in promoting meiotic apparatus assembly but also plays a regulatory role in granulosa cells, influencing maturation (He, Zhang et al. 2021).

HDAC8 is found in the cytoplasm of GV oocytes and accumulates near chromosomes after GVBD, in the spindle poles in MI and MII. Defects in these deacetylases lead to spindle defects and chromosome misalignment during meiosis (Zhang, Lu et al. 2017).

Other HDACs have also been associated with oocyte maturation. HDAC4 have high levels during oocyte growth and maturation (Kageyama, Liu et al. 2006). HDAC6 is localized in the cytoplasm of GV oocytes and associated with meiotic apparatus assembly (Zhou, Choi et al. 2017).

Additionally, sirtuins, such as SIRT1, SIRT2, SIRT3 and SIRT6, are also deacetylases important for oocyte growth and maturation in humans, contributing significantly to mitochondrial function, spindle integrity, and chromosome alignment during oocyte development (Di Emidio, Falone et al. 2014, Zhang, Hou et al. 2014, Han, Ge et al. 2015, Zhang, Han et al. 2015, Tatone, Di Emidio et al. 2018).

Histone acetylation also occurs through the addition of acetyl group onto amino terminal of the core histone proteins by histone acetyltransferases. HATs are divided into three groups: Gcn5/PCAF (general control of amino-acid synthesis 5/p300-CBP-associated factor), p300/CBP (adenoviral E1A-associated protein/CREB-binding protein) and MYST.

One of the most studied HATs is HAT8, which belongs to the MYST family. It is crucial for H4K16 acetylation and mouse oocyte development and regulates ROS levels. Its expression peaks in GV oocytes and decreases after GVBD. Deletion of KAT8 leads to infertility, impaired follicle development, and increased oocyte apoptosis (Yin, Jiang et al. 2017).

The spatiotemporal regulation of histone methylation and demethylation plays a crucial role in modulating gene expression necessary for proper oocyte maturation and early embryonic development. Various histone methylation marks, particularly on histone H3, are crucial for regulating the cellular processes necessary for successful oocyte maturation from the GV to MII stages (Bilmez, Talibova et al. 2022). In mouse oocytes, H3K4me3 accumulates not only at promoters but also in intergenic regions, and it can contribute to both transcriptional activation and repression during meiotic resumption and embryonic genome activation (Dahl, Jung et al. 2016). In human oocytes, H3K4me3 levels were found to decrease from the GV to the MII stage, however, it was not significant (Zhang, Xu et al. 2012). Overall, H3K4 methylation modifications (H3K4me1, H3K4me2, H3K4me3) play essential roles in controlling gene expression and chromatin remodelling during oocyte development.

Histone methylation modification is regulated by histone lysine methyl transferases (KMTs) and KDMs via modifying lysine residues and the number of methyl groups. SETD1, KMT2, and KDM family proteins are critical for regulating H3K4 methylation and ensuring proper oocyte development, meiotic progression, and embryonic viability (Bilmez, Talibova et al. 2022).

Protein phosphorylation also influences mitosis and meiosis by histone H3 phosphorylation at Ser10 and Ser28, affecting chromatin condensation. Even though H3/Ser10 can be phosphorylated by several kinases, Aurora B is the most important kinase during mitosis and meiosis ensuring accurate chromatin dynamics during oocyte meiosis (He, Zhang et al. 2021).

The ubiquitination and deubiquitination regulate protein degradation, cell cycle progression, and transcription, being crucial for oocyte maturation. For example, the meiotic progression is only possible due to the degradation of cyclin B and securin by the Anaphase-Promoting Complex (APC), a ubiquitin-protein ligase. Similarly, E3 ligases, such as CRL4, play a key role in maintaining oocyte survival and meiotic progression (He, Zhang et al. 2021).

Histone Glycosylation and SUMOylation are additional modifications involved in oocyte development regulation. The first one includes protein N-glycosylation and protein O-glycosylation which are associated with meiotic division and oocyte quality, respectively. The second one includes for example SUMO1, SUMO2, and SUMO3, which are involved in key events like meiotic resumption and spindle formation (Talamillo, Barroso-Gomila et al. 2020).

6.3. RNA modifications in oocytes

Oocyte growth and maturation rely on the accumulation of abundant RNAs and precise post-transcriptional regulation. Several important modifications regulate coding and non-coding RNAs, affecting their stability, translation, and function. These include N6-methyladenosine (m⁶A), (N1-methyladenosine (m¹A), 5-methylcytosine (m⁵C), N7-methylguanosine (m⁷G), 2'-O Methylation, N4-acetylcytidine (ac4C), Inosine and Pseudouridine.

During maturation, the oocyte depends on maternal RNA due to the condition of inactive transcription. Consequently, as mentioned before, the regulation of oocyte gene expression is mainly based on mRNA stability and translation level and m⁶A-RNA is an important part of this process. The m⁶A modifications are regulated by the m⁶A modulators. The formation of m⁶A is catalyzed by methyltransferase (METTL3/METTL14, WTAP, KIAA1429, RBM15 A/B, ZC3H13, and HAKAI) and erased by demethylases (FTO and ALKBH5). m⁶A can be recognized by readers, such as YTH-domainfamily proteins (YTHDFs), YTH domain-containing proteins(YTHDC1/2), IGFBP1/2/3, HNRNPs, eIF3, LRPPRC, and Prrac2 (Sun, Lu et al. 2022).

Changes in m⁶A methylation patterns occur during the transition from small to large follicles and loss of m⁶A leads to impaired gametogenesis and oocyte maturation (Cao, Zhang et al. 2020, Fang, Wang et al. 2021).

METTL3, *METTL14*, and *ALKBH5* are key genes highly expressed in oocytes, and their functions are essential for these processes (Sui, Hu et al. 2020) *METTL3* inactivation is associated with follicular development defects, and abnormal meiosis (Mu, Zhang et al. 2021). The inhibition of maturation after *METTL3* deletion occurs because mRNA translation efficiency and transcript degradation are affected (Sui, Hu et al. 2020). Additionally, *KIAA1429* is another methyltransferase necessary for folliculogenesis and oocyte competence, and its loss leads to failed GVBD and RNA metabolism defects (Hu, Ouyang et al. 2020). Another important protein is the YTHDF2, a m⁶A reader, that regulates maternal RNA degradation during oocyte maturation (Ivanova, Much et al. 2017). Additionally, YTHDC1 is a m⁶A reader that intervenes in pre-mRNA processing (Kasowitz, Ma et al. 2018). These and other genes collectively ensure proper m⁶A methylation, influencing oocyte maturation, RNA stability, and fertility

RESEARCH AIMS

The primary aim of this thesis was to understand the molecular mechanisms underlying human oocyte meiotic maturation, with a focus on transcriptomic and epigenetic dynamics. Specifically, the following aims were addressed:

- Characterization of the transcriptional landscape of human oocytes across meiotic maturation stages, identifying and quantifying genes expressed at Germinal Vesicle (GV), Metaphase I (MI), and Metaphase II (MII) stages. Additionally, determine stage-specific genes and their associated biological processes.
- Investigate the role of epigenetic regulators during meiotic maturation, observing the expression profiles of key epigenetic regulators and also the oocyte localization of some epigenetic regulators and epigenetic modifications (5mC and 5hmC).

By focusing on these aims, we seek to provide novel insights into the determinants of oocyte quality, enhancing the understanding of reproductive biology

MATERIAL AND METHODS

1. Ethical considerations

This study was rigorously reviewed and received formal approval from the Ethics Committee for Health (CES) of the Hospital de São João (CHSJ) in collaboration with the Faculty of Medicine of the University of Porto (FMUP), under approval reference CE 429-19.

Each participant provided signed informed consent for oocyte donation. To maintain the highest levels of privacy and data protection, all biological materials used in this research were donated anonymously, with no personal identifiers attached to any samples. Data handling protocols were implemented to securely anonymize and manage all biological samples and personal information, allowing the research to be conducted with strict adherence to ethical and legal requirements for participant protection.

2. Study cohort

The study involved fresh oocytes from donors who underwent ovarian stimulation at IVF clinics in Porto, specifically at the Centro de Genética da Reprodução Prof. Alberto Barros (CGR) and PROCRIAR. All donors were carefully selected based on strict criteria: each had fewer than four-lifetime donations, and all were between 18 and 34 years old. Following this initial screening, donor eligibility was confirmed by the National Council for Medically Assisted Procreation (CNPMA), after which each donor underwent comprehensive evaluations.

These evaluations included a gynaecological examination and a psychological assessment to confirm physical and mental awareness for oocyte donation. Additionally, donors provided blood samples for an extensive series of tests, including a complete blood count, biochemical profile, serology, and screening for infection markers such as Syphilis (VDRL), Cytomegalovirus (CMV), hepatitis B and C, and human immunodeficiency virus (HIV) types 1 and 2. Blood typing, Rhesus (RH) factor determination, and hormonal assessments were also conducted.

Furthermore, genetic screening was performed, including karyotyping and tests for genetic disorders such as cystic fibrosis, Fragile X syndrome, and spinal muscular atrophy, to ensure the absence of pathogenic variants in the donor population. All oocyte donor characteristics are presented in Table 2 and 3, with no statistically significant differences observed regarding Age, Body Mass Index (BMI) or Smoking status.

3. Ovarian stimulation

Oocyte donors underwent controlled ovarian hyperstimulation with a gonadotropin releasing hormone (GnRH) antagonist protocol. This protocol consists of the administration of ganirelix (Orgalutran, 0.25 mg; MSD, Hertfordshire, UK) to prevent premature ovulation. For

ovarian stimulation, recombinant follicle-stimulating hormone (rFSH) was used, mainly follitropin alfa (Gonal-F; Merck Serono, Geneva, Switzerland) and rarely with Elonva;(Organon, Kloosterstraat, Netherlands). rFSH stimulation usually began on the 2nd or 3rd day of the menstrual cycle and was adjusted according to the ovarian response monitored by transvaginal ultrasound and serum estradiol levels. The stimulation period varied between 8 and 12 days, depending on the follicular development observed.

Ovulation induction occurs after confirming that at least three dominant follicles have reached a diameter of 17 mm (or, in cases with a lower number of growing follicles, at least one dominant follicle). For induction of maturation, a GnRH agonist (0.2 mg, triptorelin, Decapeptyl; Ipsen Pharma Biotech, Signes, France) was administered 36h before ultrasound-guided transvaginal oocyte retrieval. This medication promotes an LH surge that allows the final process of oocyte maturation to take place.

4. Oocyte retrieval and denudation

The size of the follicles was monitored over several days before the follicle puncture, which allowed the optimal time for the puncture to be established (Table 1). Therefore, different sizes of follicles were aspirated in the puncture.

The follicles were manually aspirated with a Cook Needle, with guidance from a transvaginal ultrasound in order to visualise the ovaries and follicles in real time. This allows for precise guidance during the procedure and correct follicular aspiration.

Immediately afterwards, the oocyte handling was performed in Origio media (Origio, Malöv, Denmark) in a heated airflow (K-system, Kivex Biotech, Denmark; Cooper Surgical, Malöv, Denmark). After oocyte collection, the oocytes were incubated at optimal conditions, 5% O₂ 6% CO₂, 37°C, until cumulus cells denudation.

Denudation was performed enzymatically with Cumulase (Origio) and then mechanically in SPM (Origio) with Handling pipettes (Vitrolife), approximately 3 hours after the puncture.

After denudation, oocytes were placed in a tube with 3µL of FERT medium (Origio) and immediately stored at -20°C for a maximum of 2 hours.

The oocytes were then snap-frozen and incubated at -80°C after adding 2.5µL of Buffer RLT (Qiagen).

Table 1 Number and size of follicles detected by ultrasound for each donor (48h before oocyte collection) and number of oocytes obtained on the day of the follicle puncture.

Sample	Donor	Right ovary		Left ovary		Number of oocytes		
		N	Diameter (mm)	N	Diameter (mm)	GV	MI	MII
7-MII	4	1	21	1	20	0	0	8
		3	20	3	18			
		1	19	1	17			
		1	18	3	13			
		2	17					
11-GV	5	1	19	1	20	2	0	29
		1	18	2	18			
		2	17	2	17			
		2	16	1	16			
		1	15	2	15			
		4	14	4	14			
		1	13	2	13			
		3	12	1	12			
		1	11	3	10			
		2	10					
12-MII 13-MII	6	1	9			0	0	13
		1	18	1	20			
		1	16	3	16			
		2	15	1	15			
		2	14	1	13			
		3	10					
15-MI 16-MII 17-MII	7	1	8			0	1	29
		2	17	1	18			
		3	16	3	17			
		3	15	1	16			
		3	14	3	15			
		6	13	4	14			
		2	12	2	13			
		2	11	2	12			
		3	10	1	11			
				2	10			
36-MI 37-GV	14	1	16	1	19	1	1	11
		1	15	1	17			
		1	13	3	16			
		1	11	1	9			
		3	10					
		1	9					
38-GV	15	1	19	1	20	1	0	18
		3	18	1	19			
		2	17	2	18			
		2	16	2	17			
		2	14	1	16			
		1	10	2	15			
		1	8	3	14			
				2	13			
				1	10			
				1	8			
45-MI	18	1	18	1	19	1	1	16
		2	17	1	18			
		1	16	2	17			
		1	15	1	16			

		1	14	1	15			
		1	13	1	14			
		1	12	1	13			
		1	11	1	10			
		1	10	1	9			
		1	9	1	6			
		1	8	1	5			
47-MI 48-MI 50-GV	19	1	20	1	18	2	5	10
		1	18	1	17			
		3	16	2	16			
		5	15	6	14			
		2	14	3	13			
		1	13	3	12			
		2	12	2	11			
		3	11	2	10			
		1	10	1	8			
		1	9					
		1	8					
52-MI 53-MII	20	1	19	2	21	0	1	13
		1	18	3	20			
		1	14	2	19			
		1	13	3	16			
		2	11	3	15			
		1	6	2	13			
54-GV 55-GV	21	1	23	1	16	5	0	10
		1	21	3	14			
		1	20	1	13			
		1	18	3	12			
		1	14	1	11			
		2	13	2	10			
		1	12	8	6			
		1	11					
85-MII	29	8	6			0	0	12
		2	16	1	20			
		7	14	2	18			
		4	13	1	17			
		2	12	1	16			
				1	15			
				2	14			
				2	13			
				2	12			

Human oocytes were classified into three distinct maturation stages, according to their morphological characteristics, namely, the presence of an intact and visually distinct nuclear membrane and prominent nucleolus (Germinal Vesicle – GV), the presence of an extruded first polar body (Metaphase II – MII) and a homogeneous cytoplasm (Metaphase-MI) (Fig. 3). Abnormal-appearing oocytes, with signs of degeneration, were excluded from this study. All the oocytes used in this research were surplus material that would otherwise be discarded, including MII oocytes in excess.

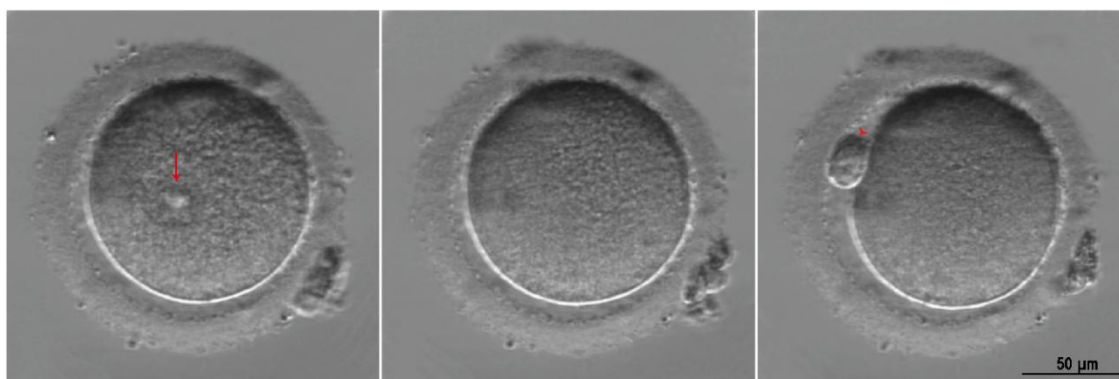


Figure 3 Human oocyte maturation stages. Phase-contrast microscopy images of human oocytes at each maturation stage: GV – Germinal-Vesicle; MI – Metaphase I; MII – Metaphase II. Arrow – nuclear envelope in GV oocyte; Arrowhead – first polar body in MII oocyte.

5. Single-oocyte RNA sequencing

5.1. Sample selection

To minimize the impact of external variables, transcriptome analysis was conducted on oocytes collected from a single IVF clinic (CGRAB). A total of 19 oocytes were individually analyzed, comprising 6 at the Germinal Vesicle (GV) stage (Prophase I of the cell cycle), 6 at Metaphase I (MI), and 7 at Metaphase II (MII). These oocytes were obtained from eleven distinct donors, with each donor contributing oocytes from a single cycle of ovarian stimulation. Detailed information regarding the donors and follicle sizes prior to oocyte retrieval is available in Tables 2 and 3.

Table 2 - Characteristics of human oocyte donors.

Oocyte RNA-Seq						
(Number of donors n=11)						
	GV (n=6)	MI (n=6)	MI (n=7)	p-value MI vs GV	p-value MII vs MI	p-value MII vs GV
Age, mean (SD)	25.5 (3.8)	25.8 (4.6)	26.0 (4.8)	0.894	0.950	0.841
IMC (SD)	23.5 (3.7)	20.3 (2.1)	20.9 (3.2)	0.099	0.074	0.195

GV- Germinal vesicle oocyte, MI- Metaphase I oocyte, MII- Metaphase II oocyte, SD- Standard deviation.

Statistical analysis - t-test

Table 3 Detailed characteristics of donors for each single oocyte analysed by RNA-Seq.

Sample	Donor	Collection date	Stage	Donor age	Mean age (SD)	BMI	Mean BMI (SD)	Smoking status
11-GV	5	11/18/2020	GV	25	25.5 (3.8)	30	23.5 (3.7)	No
37-GV	14	9/30/2021	GV	33		24		No
38-GV	15	10/1/2021	GV	22		24		No
50-GV	19	1/20/2022	GV	25		19		No
54-GV	21	2/14/2022	GV	24		22		Yes (3/day)
55-GV	21	2/14/2022	GV	24		22		Yes (3/day)
15-MI	7	12/3/2020	MI	20	25.8 (4.6)	19	20.3 (2.1)	No
36-MI	14	9/30/2021	MI	33		24		No
45-MI	18	12/13/2021	MI	23		22		Yes (3/day)
47-MI	19	1/20/2022	MI	25		19		No
48-MI	19	1/20/2022	MI	25		19		No
52-MI	20	2/2/2022	MI	29		19		No
7-MII	4	11/9/2020	MII	23	26.0 (4.8)	28	20.9 (3.2)	No
12-MII	6	11/23/2020	MII	30		20		Yes (5-6/day)
13-MII	6	11/23/2020	MII	30		20		Yes (5-6/day)
16-MII	7	12/3/2020	MII	20		19		No
17-MII	7	12/3/2020	MII	20		19		No
53-MII	20	2/2/2022	MII	29		19		No
85-MII	29	7/5/2022	MII	30		21		No

GV- Germinal vesicle oocyte, MI- Metaphase I oocyte, MII- Metaphase II oocyte, SD- Standard deviation.
Statistical analysis - t-test (Age and BMI).

5.2. cDNA synthesis and library preparation

Full-length single-oocyte RNA sequencing (soRNA-seq) libraries were generated at the Genomics Unit of the Gulbenkian Institute of Science (IGC, Portugal). The process adhered closely to the protocol established by (Macaulay, Teng et al. 2016), ensuring high-quality transcriptome data from individual oocytes.

Each single oocyte was collected and immediately preserved in RLT buffer (Qiagen) to stabilize RNA and prevent degradation. This step is critical to maintaining RNA integrity, which is essential for high-quality sequencing results.

The RNA from each oocyte was processed for full-length cDNA synthesis according to the detailed protocol described by Macaulay and collaborators. In this step, RNA molecules were reverse transcribed into complementary DNA (cDNA). The protocol involved capturing polyadenylated (polyA+) mRNA using oligo-dT coated magnetic beads.

Reverse transcription was carried out with a reverse transcriptase enzyme and a template Switching Oligo (TSO) to ensure the synthesis of complete transcripts.

Quality control of the synthesized cDNA was performed using a Fragment Analyzer (Agilent Technologies) to verify fragment size distribution and cDNA integrity.

Library preparation involved the following key steps:

Library preparation including cDNA 'tagmentation', PCR-mediated adaptor addition and amplification of the adaptor-ligated libraries was performed following the Nextera library preparation protocol (Nextera XT DNA Library Preparation kit, Illumina), as described by Baym and collaborators (Baym, Kryazhimskiy et al. 2015).

"Tagmentation" of the cDNA consists of the fragmentation of the cDNA and the addition of adaptor sequences. Subsequently, a PCR amplification was performed to add sequencing adaptors. Purification of the amplified libraries using AMPure XP magnetic beads (Beckman Coulter) was executed to remove contaminants and ensure high-quality libraries. Additional quality control of the libraries was conducted using the Fragment Analyzer to confirm the presence and quality of the expected fragments.

Amplified libraries were sequenced in a NextSeq2000 (Illumina Inc) using 100 SE P2 to an average sequencing depth of 20 million reads per cell. Sequence information was extracted in FastQ format, using Illumina DRAGEN FASTQ Generation v3.8.4.(Table 4)

These FastQ files were then used for downstream transcriptome analysis, including the identification of gene expression profiles and differential expression across the maturation stage.

Table 4 Overview of the samples and sequencing data.

	Samples	Donor	cDNA (ng/μl)	RUN	Number of mapped reads	Number of reads aligned to genes	Mean number of mapped reads (SD)	Mean number of reads aligned to genes (SD)
GV	11-GV	5	7.305	2	37 275 647	25 482 579	35 941 444	24 939 222
	37-GV	14	9.404	2	33 111 808	23 430 031	(4 494 334)	(3 608 662)
	38-GV	15	4.336	2	35 277 163	21 800 668		
	50-GV	19	8.396	2	41 597 884	29 481 808		
	54-GV	21	3.355	2	40 228 690	29 462 493		
	55-GV	21	1.840	3	28 157 472	19 977 755		
MI	15-MI	7	4.338	2	33 549 694	20 508 886	36 440 888	26 065 652
	36-MI	14	4.622	2	35 035 935	24 955 372	(4 680 566)	(4 048 198)
	45-MI	18	9.478	2	40 884 985	29 624 192		
	47-MI	19	3.889	2	41 648 903	30 325 997		
	52-MI	20	1.077	2	39 235 414	29 657 464		
	48-MI	19	2.296	3	28 290 399	21 322 002		
MII	7-MII	4	0.938	1	35 435 118	23 511 089	33 104 937	23 101 809
	12-MII	6	2.305	2	38 366 701	25 100 139	(8 006 271)	(5 542 620)
	16-MII	7	4.791	2	36 527 921	23 494 860		
	53-MII	20	0.779	2	35 466 950	25 922 034		
	13-MII	6	3.860	3	22 463 334	15 863 479		
	17-MII	7	4.619	3	19 877 544	15 277 051		
	85-MII	29	0.277	3	43 596 989	32 544 014		

GV- Germinal vesicle oocyte, MI- Metaphase I oocyte, MII- Metaphase II oocyte, SD- Standard deviation.

5.3. Sequencing data Processing

An initial quality check of raw sequencing data was performed using FastQC (Babraham BioInformatics) (Version 0.12.0) and was considered of good quality (99% > Q30). Reads were then mapped to the human genome (GRCh38) with Hisat2 v2.2.2 (Kim, Langmead et al. 2015). Gene expression quantification was carried out using FeatureCounts (Version 2.0.3)(Liao, Smyth et al. 2014) and Kallisto (Version 0.50.0) (Bray, Pimentel et al. 2016) using gene annotations from Ensembl version 106 (April 2022) in order to compare and validate the results obtained with the two bioinformatic tools. Expression values were normalized to CPM (Counts Per Million) using the Trimmed Mean of the M-Values (TMM)

approach (Robinson, McCarthy et al. 2010) as available in the edgeR (Version 3.32.1) package from the R software (Version 4.0.5).

5.4. Bioinformatic analysis

Principal component analysis (PCA) was performed with normalized CPM values to assess the overall similarity between samples, using the R package PCAtools (Version 2.2.0). R (Version 4.0.5) was also used to plot a heatmap representing similarity between samples using the Euclidean distance of normalized gene expression (Love, Huber et al. 2014).

The expressed genes were determined considering the normalized CPM value, which should be greater than 1 (or log2CPM greater than 0) in all samples of the group. Genes uniquely expressed in each phase were defined as genes whose normalized CPM value was greater than 1 in all samples from only one of the stages. Differentially Expressed Genes (DEGs) were identified by comparing all oocyte maturation stages (GV vs MI; GV vs MII; MI vs MII). DEGs were identified using the quasi-likelihood F-test (Lun, McCarthy et al. 2016) as available in the edgeR (Version 3.32.1) package in R (Version 4.0.5). Genes with $FDR \leq 0.05$ and FoldChange (FC) $> |2|$ were assigned as differentially expressed. As an alternative and less restrictive tool, DESeq2 (Version 1.30.1) (Love, Huber et al. 2014) was also utilized to extract DEGs, using a p-adjusted value < 0.05 and $\log_2FC > |1|$.

KEGG pathway (Kyoto Encyclopedia of Genes and Genomes; <http://www.genome.jp/kegg/>) and Gene ontology (GO) analysis (<http://geneontology.org>) were performed using the tool g:GOST provided by GProfiler (Raudvere, et al. 2019) (Version e111_eg58_p18_f463989d). GO terms with an adjusted p-value < 0.05 were considered significant. Venn Diagrams were constructed using the VIB/UGent Bioinformatics and Evolutionary Genomics tool - <https://bioinformatics.psb.ugent.be/webtools/Venn/> and jvenn - <https://jvenn.toulouse.inrae.fr/app/index.html> (Bardou, Mariette et al. 2014).

Volcano plots were plotted to visualize the differentially expressed genes in each comparison using the package EnhancedVolcano (Version 1.8.0) from R. Violin plots were designed using R software and ggplot2 package (Version 3.3.6).

6. Gene expression analysis by quantitative RT-PCR (qRT-PCR)

RNA extraction from human oocytes was performed using Power SYBR® Green Cells-to-Ct™ Kit (Invitrogen), allowing reverse transcription (RT) and real-time PCR directly from cell lysates.

The Power SYBR™ Green Cells-to-CT™ kit enables rapid and efficient gene expression analysis by combining cell lysis, reverse transcription, and quantitative PCR (qPCR) in an optimised workflow. This protocol eliminates the need for RNA isolation, making it ideal for

analyzing gene expression directly from cells with minimal processing time and potential RNA loss.

Briefly, we added 50µL of lysis solution (with DNase I) to the single oocyte and incubated at room temperature (RT) for 5 minutes. Then, 5µL of stop solution was added to the lysate and incubated for 2 minutes at RT. Reverse transcription was performed by mixing 10µL of the lysate with 25µL of SYBR RT Buffer and 2.5µL of RT Enzyme Mix, for a final volume of 50µL. Afterwards, the mixture was incubated at 37°C for 60 min, and then at 95°C for 5 min to inactivate the RT enzyme.

Real-time PCR was performed using DNA-binding dye PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Therefore, we mixed 5µL of 2xPowerUp mix, 0,25µL of each primer (at 20µM) and 2µL of the cDNA, for a final volume of 10µL. Then, the mix was incubated in the thermocycler according to the following program (50°C for 2min; 95°C for 2 min and 40X the following 95°C for 3 secs, 60°C for 30 secs). Melt curve was also performed after the amplification.

Specific primers were designed for amplification of *DNMT1* AND *ZFAND2A*. Additionally, *UBB* was used as a reference gene (Table 5). We analysed the expression of the following genes: *DNMT1* in 9 GV oocytes and 4 MII oocytes, *ZFAND2A* in 10 GV oocytes and 3 MII oocytes. Statistical analysis was performed using the Mann-Whitney U test, with the Statistical Package for Social Sciences (SPSS, IBM) software v29.0.

Table 5 Primers used in RT-qPCR.

Gene	Sequence 5'→3'	Size(bp)	Ta(°C)	Reference
DNMT1 Fw	TGGACGACCCTGACCTCAAA	168	60	Marques et al. 2011
DNMT1 Rv	TGCTTACAGTACACACTGAAGCAG		60	Marques et al. 2011
UBB Fw	TCCGCTAACAGGTCAAAATGCA	129	60	Marques et al. 2011
UBB Rv	GGGAATGCCTTCCTTATCCTG		60	Marques et al. 2011
ZFAND2A Fw	TGCAAGCAGCTAGATTTTCTTCC	125	60	Unpublished
ZFAND2A Rv	ACTGGGACGTGAACATCCTTCT		60	Unpublished

Bp-base pair; Fw- Forward; Rv-Reverse

7. Immunostaining of 5mC and 5hmC-in oocytes

Single oocytes were washed in 1x PBS and fixed in 4% paraformaldehyde (PFA) in PBS, for 10 min at room temperature. Then, oocytes were washed with 1x PBS and stored in PBT (PBS 0.05% Tween-20) with 0.05% Sodium Azide, at 4°C until further processed. Immunostaining of DNA methylation (Monoclonal antibody: Invitrogen; RM231) and DNA

hydroxymethylation (Monoclonal antibody: Active Motif, #40000) was performed as described (Santos, Peat et al. 2013). Briefly, oocytes were washed in PBS and permeabilized with 0.5% Triton X-100 for 1 hr at room temperature. After washing with PBT, oocytes were treated with 4 N HCl 0.1% Triton X-100 for 10 min at room temperature, washed in PBT and blocked overnight with PBS 0.05% Tween-20 1%BSA (BS). After, incubation with the primary antibody (1:500 5hmC and 5mC) for 1 hour followed by 30 min wash with BS and incubation with secondary antibody for 5mC (rabbit anti-mouse AlexaFluor 594, #A11012, Molecular Probes) and for 5hmC (goat anti-mouse AlexaFluor 488, #A11029, Molecular Probes) for 1h in the dark. After washing with BS for 30 min, slides were mounted with Vectashield mounting medium with DAPI (Vector Labs) and stored at 4°C until observed in a fluorescence microscope (Zeiss Axio Imager.Z1).

Immunostaining of DNMT1 (Monoclonal antibody: Cell Signaling, D63A6) and TET3(Monoclonal antibody: Sigma, HPA050845) was also performed similarly with the protocol mentioned above however without the 4 N HCl 0.1% Triton X-100 treatment.

Briefly, oocytes were washed in PBS and permeabilized with 0.5% Triton X-100 for 1 hr at room temperature. After washing with PBT, oocytes were blocked overnight with PBS 0.05% Tween-20 1%BSA (BS). After, incubation with the primary antibody (1:100 DNMT1 and 1:500 TET3) for 1 hour followed by 30 min wash with BS and incubation with secondary antibody (rabbit anti-mouse AlexaFluor 594, #A11012, Molecular Probes) for 1h in the dark. After washing with BS for 30 min, slides were mounted with Vectashield mounting medium with DAPI (Vector Labs) and stored at 4°C until observed in a fluorescence microscope (Zeiss Axio Imager.Z1).

Images were taken LSM 510 Meta confocal laser scanning microscope (Carl Zeiss).

8. Data availability

The datasets and codes generated in this study were deposited in Zenodo (doi: 10.5281/zenodo.14163313). All the other data are available from the corresponding author upon reasonable request.

RESULTS

1. Transcriptome analysis of human oocytes

We isolated single human oocytes and classified them based on their morphological characteristics into three distinct developmental stages: Germinal Vesicle (GV), Metaphase I (MI), and Metaphase II (MII) (Fig. 3), as detailed in the Materials and Methods section. Subsequently, we conducted a transcriptomic analysis using single-oocyte RNA sequencing (soRNA-Seq). In total, 19 oocytes from 11 donors were sequenced individually, comprising 6 GV, 6 MI, and 7 MII oocytes.).

1.1. Principal component analysis and distance heatmap

Principal Component Analysis (PCA) was performed following gene expression quantification with FeatureCounts (Liao, Smyth et al. 2014). The analysis revealed relatively dispersed samples; however, a degree of clustering based on oocyte maturation stages was evident. GV oocytes, in particular, clustered predominantly in the upper-left quadrant and were more distant from the MI and MII stages (Fig. 4A).

The group of MI oocytes likely comprises oocytes that are in the process of transitioning to the MII stage but have not yet completed polar body extrusion, thereby exhibiting a gene expression profile more closely aligned with MII oocytes. Conversely, MII oocytes demonstrated a lower degree of aggregation in the PCA plot and in the distance heatmap (Fig. 4B), which may be indicative of increased heterogeneity within this group, potentially reflecting biological variability or subtle differences in maturation status among the samples.

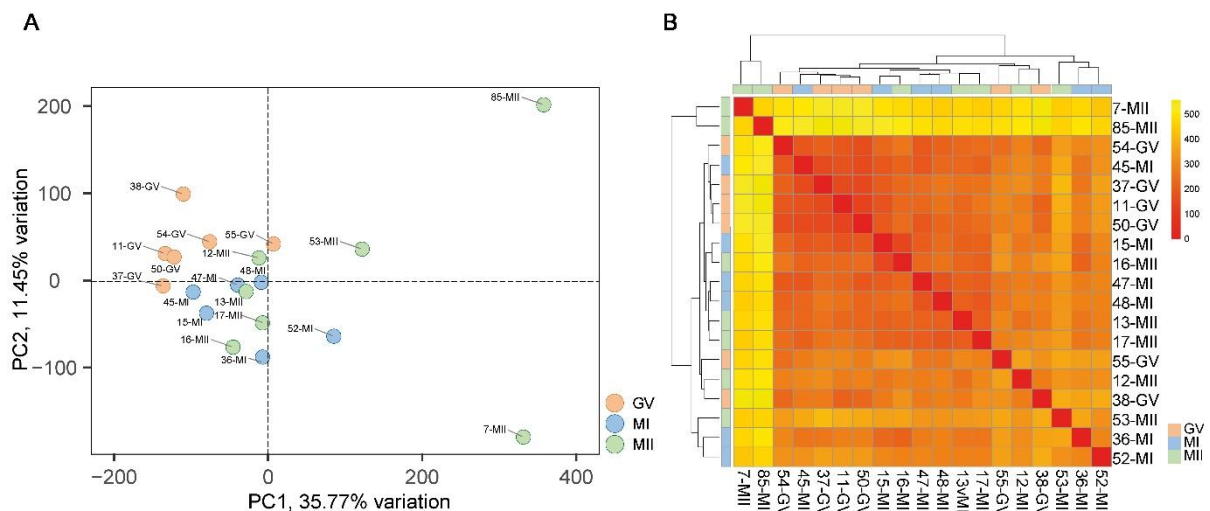


Figure 4 A) Principal Component Analysis (PCA) plot of all oocytes quantified with FeatureCount.. GV oocytes are represented in orange, MI oocyte in blue and MII in green and B) Heatmap showing the correlation coefficient between transcriptomes of all oocytes, reflecting similarities and differences in gene expression across the maturation stage

To evaluate the robustness of our transcriptomic analysis, we compared two different tools for gene expression quantification: FeatureCounts and Kallisto (Bray, Pimentel et al. 2016). FeatureCounts is a read-counting method that maps reads to annotated gene features and is widely used for quantifying gene expression at the gene level. In contrast, Kallisto employs a pseudo-alignment approach that quantifies transcript-level abundance. Despite these methodological differences, both tools yielded consistent results, with PCA plots showing similar clustering patterns according to oocyte maturation stages and heatmaps reflecting comparable distance relationships between samples (Fig.4 and Fig.5). This agreement between methods supports the reliability of our findings and suggests that the observed biological trends are robust to the choice of quantification tool.

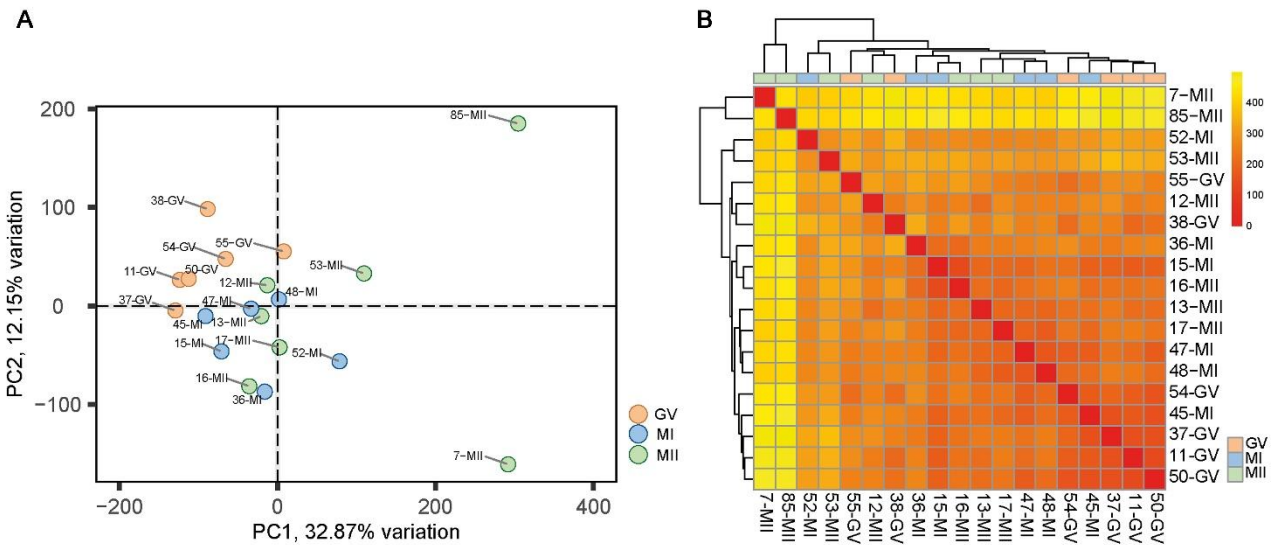


Figure 5 A) Principal Component Analysis (PCA) plot of all oocytes quantified with Kallisto. GV oocytes are represented in orange, MI oocyte in blue and MII in green and B) Heatmap showing the correlation coefficient between transcriptomes of all oocytes, reflecting similarities and differences in gene expression across the maturation stage

Therefore, we performed the remaining analyses using FeatureCounts for gene expression quantification, due to its accuracy in assigning reads to annotated gene features and its widespread use in transcriptomic studies. This approach ensures reliable and reproducible results, particularly when analyzing gene expression data.

1.2. Expressed genes during oocyte maturation

To determine the genes expressed at each oocyte maturation stage, we selected transcripts with a CPM (Counts Per Million) value greater than 1 (or $\text{Log}_2\text{CPM} > 0$) across all samples within each group. Using this threshold, we identified 9,660 expressed genes in GV oocytes, 8,734 in MI oocytes, and 5,889 in MII oocytes. The top 10 most highly expressed genes at each maturation stage are presented in Table 6

Among these, *TUBB8* (Tubulin Beta 8 Class VIII) emerged as the most highly expressed gene across all maturation stages, accompanied by other members of the same gene family, such as *TUBB8P7* and *TUBB8B*. Furthermore, mitochondrial genes, including *MT-ND4* and *MT-CO1*, exhibited consistently high expression levels across all stages, as did *Histone H3-3B*.

Other genes demonstrating robust expression across all maturation stages included *DPPA3* (Developmental Pluripotency Associated 3), *WEE2* (WEE2 Oocyte Meiosis Inhibiting Kinase), *OOSP2* (Oocyte Secreted Protein 2), the ZP (Zona Pellucida) gene family, *BMP15* (Bone Morphogenetic Protein 15), and *GDF9* (Growth Differentiation Factor 9) (Table 6).

Table 6 Top 10 expressed genes at each oocyte maturation stage. GV – Germinal-Vesicle (Prophase I); MI – Metaphase I; MII – Metaphase II.

GV		MI		MII	
Gene	Expression	Gene	Expression	Gene	Expression
<i>TUBB8</i>	12.83	<i>TUBB8</i>	14.15	<i>TUBB8</i>	14.32
<i>GDF9</i>	12.70	<i>H3-3B</i>	12.98	<i>H3-3B</i>	13.68
<i>MT-ND4</i>	12.14	<i>TUBB8P7</i>	12.92	<i>DPPA3</i>	13.22
<i>H3-3B</i>	12.04	<i>MT-CO1</i>	12.87	<i>TMSB4X</i>	13.12
<i>ZP3</i>	12.03	<i>MT-ND4</i>	12.79	<i>MT-CO1</i>	13.11
<i>MT-CO1</i>	11.85	<i>TUBB8B</i>	12.73	<i>MT-ND4</i>	13.03
<i>TUBB8P7</i>	11.80	<i>DPPA3</i>	12.71	<i>TUBB8B</i>	12.95
<i>TCL1A</i>	11.78	<i>GDF9</i>	12.61	<i>WEE2</i>	12.93
<i>TUBB8B</i>	11.67	<i>WEE2</i>	12.55	<i>TUBB8P7</i>	12.88
<i>TUBA1C</i>	11.64	<i>ACTL8</i>	12.54	<i>PTTG1</i>	12.85

Expression values are the mean of Log2 CPM of all the samples analysed at each stage.

Gene Ontology (GO) analysis of the highly expressed genes (Log2CPM > 10) at each maturation stage revealed overlapping terms across all three oocyte stages, primarily related to mitochondrial function and oocyte development and maturation (Fig. 6).

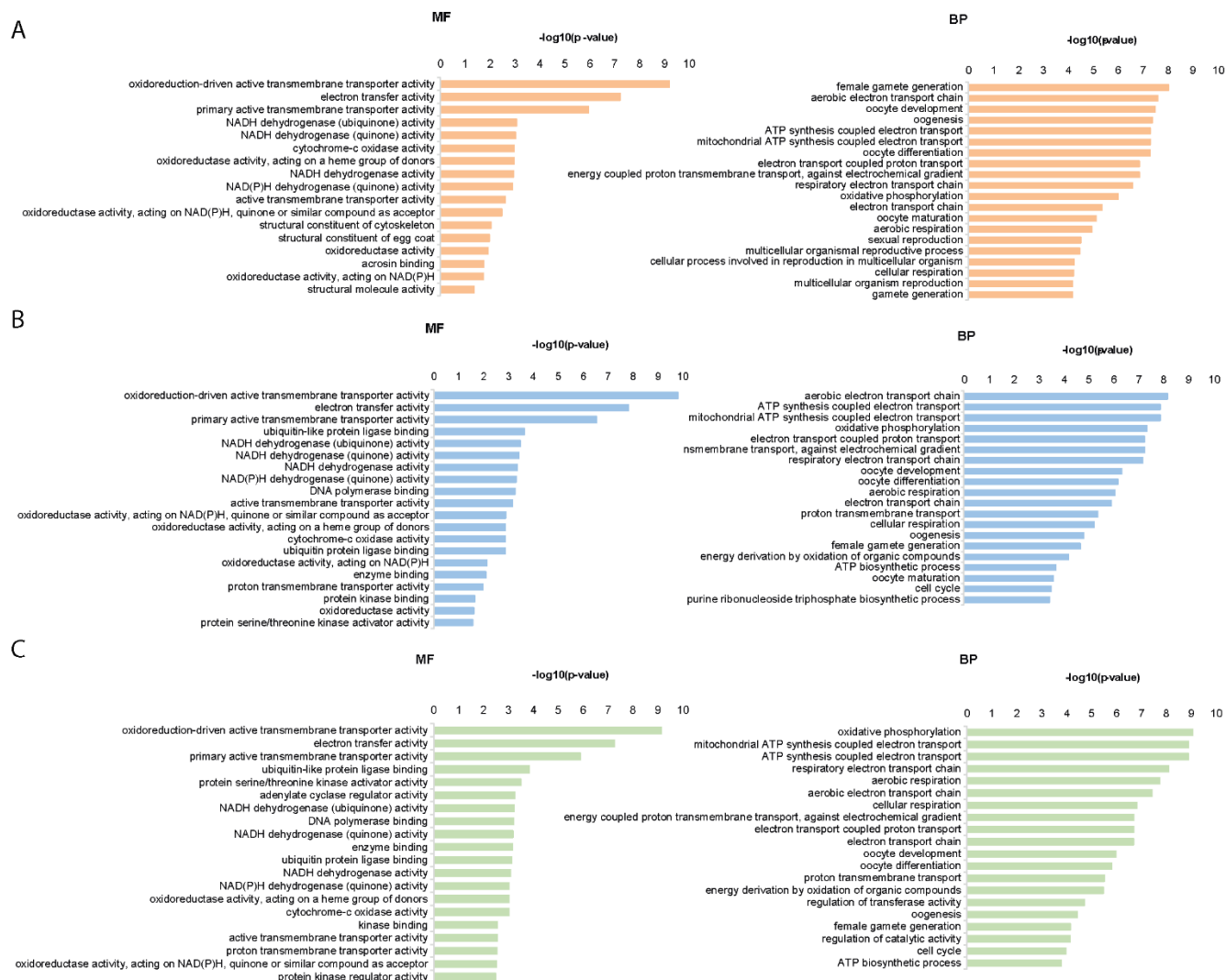


Figure 6 Gene Ontology (GO) analysis of highly expressed genes ($\log_2\text{CPM} > 10$) in each stage of oocyte maturation. Top20 significant terms for molecular function (MF) and biological processes (BP) in (A) Germinal vesicle (GV; orange bars), (B) Metaphase I (MI; blue bars) and (C) Metaphase II (MII; green bars).

Further analysis showed that among the expressed genes, 5602 were common to all three maturation stages, while 1328 were unique to GV, 401 to MI, and 72 to MII oocytes (Fig.7A). GV and MI shared the highest number of genes ($n = 2623$), whereas MI and MII shared 108 genes, and GV and MII shared 107 genes (Fig. 7A).

GO analysis of the 5602 genes expressed across all maturation stages highlighted terms associated with organelle organization, metabolic and biosynthetic processes, and cell cycle regulation (Fig. 7B). Regarding molecular function, protein binding was the most significant term, followed by catalytic activity and enzyme binding.

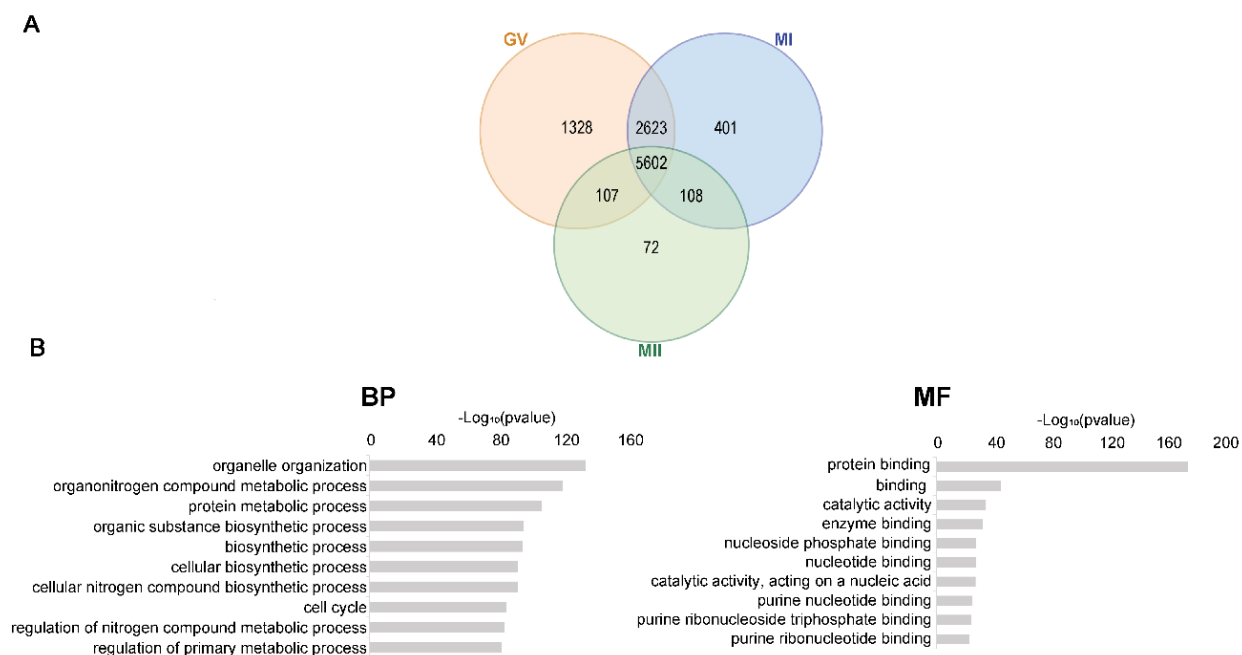


Figure 7 A) Venn Diagram showing the number of unique and shared genes in each oocyte maturation stage; B) Gene Ontology (GO) analysis of the 5602 genes commonly expressed in all maturation stages, highlighting the most significant terms associated with biological processes (BP) and molecular functions (MF). GV – Germinal-Vesicle (Prophase I); MI – Metaphase I; MII – Metaphase II. Gene Ontology biological processes (BP) and molecular function (MF) terms.

For uniquely expressed genes, particularly in GV oocytes, GO analysis revealed protein binding as the most enriched term, followed by catalytic activity, ion binding, and transferase activity (Table 5). Notably, the molecular function terms “S-adenosylmethionine-dependent methyltransferase activity” and “methyltransferase activity” were identified, as potentially linked to epigenetic reprogramming events during oocyte development (Qian and Guo 2022).

In MI oocytes ($n = 401$), only two statistically significant GO terms were identified: “biological regulation” and “regulation of biological process”. In contrast, no statistically significant GO terms were found for the uniquely expressed genes in MII oocytes, likely due to the small number of genes analyzed (Table 7).

Table 7 Gene Ontology analysis (top 10) of uniquely expressed genes for each maturation stage. GV – Germinal-Vesicle (Prophase I); MI – Metaphase I; MII – Metaphase II.

GV			
GO	Term	N° of genes	p-value
MF	protein binding	1020	3.16E-17
MF	catalytic activity	449	1.27E-08
MF	transferase activity	206	4.70E-06
MF	ion binding	457	8.99E-06
MF	small molecule binding	463	1.10E-04
MF	metal ion binding	331	3.61E-04
MF	cation binding	334	9.51E-04
MF	transferase activity, transferring one-carbon groups	34	1.39E-03
MF	S-adenosylmethionine-dependent methyltransferase activity	30	1.61E-03
MF	methyltransferase activity	32	4.00E-03
BP	organonitrogen compound metabolic process	430	5.74E-09
BP	ncRNA processing	55	1.35E-05
BP	cellular nitrogen compound biosynthetic process	343	1.90E-05
BP	organelle organization	265	1.10E-04
BP	small molecule metabolic process	149	3.36E-04
BP	ncRNA metabolic process	67	4.46E-04
BP	macromolecule modification	240	4.64E-04
BP	catabolic process	196	4.82E-04
BP	mitochondrion organization	55	8.64E-04
BP	mitochondrial respiratory chain complex assembly	20	1.59E-03
MI			
GO	Term	N° of genes	p-value
BP	biological regulation	247	2.49E-03
BP	regulation of biological process	240	4.62E-03

1.3. Differential expression analysis between maturation stages

To acquire insights into the transcriptional differences between oocytes in different maturation stages, we performed differential expression analysis between the three maturation stages: GV, MI, and MII. By comparing transcript abundance across stages, we identified genes with statistically significant differences in expression levels, highlighting key molecular processes associated with oocyte development.

Our analysis revealed distinct sets of upregulated and downregulated genes for each pairwise comparison, reflecting stage-specific transcriptional dynamics.

To identify transcriptomic changes associated with oocyte maturation, we extracted Differentially Expressed Genes using a $FC > |2|$ (or $\log_2FC > |1|$) and a $FDR < 0.05$.

Using two bioinformatic tools, EdgeR (Lun, McCarthy et al. 2016) and DESeq2 (Love, Huber et al. 2014), we analyzed differentially expressed genes (DEGs) between oocyte maturation stages. With EdgeR, we identified 42 DEGs between GV and MI stages and 1165 DEGs between GV and MII stages; notably, no DEGs were detected between MI and MII stages. In contrast, DESeq2 identified 403 DEGs between GV and MI, 2981 DEGs between GV and MII, and 80 DEGs between MI and MII, using a threshold of adjusted $p\text{-value} < 0.05$ and $|\log_2FC| > 1$.

Comparison of the results from both tools revealed that EdgeR applies a more stringent filtering, capturing fewer DEGs than DESeq2. Importantly, nearly all DEGs identified by

EdgeR were also detected in the DESeq2 analysis (Fig.8), demonstrating the robustness of the results. Given the higher stringency of EdgeR, we opted to use DEGs identified by this tool for subsequent analyses.

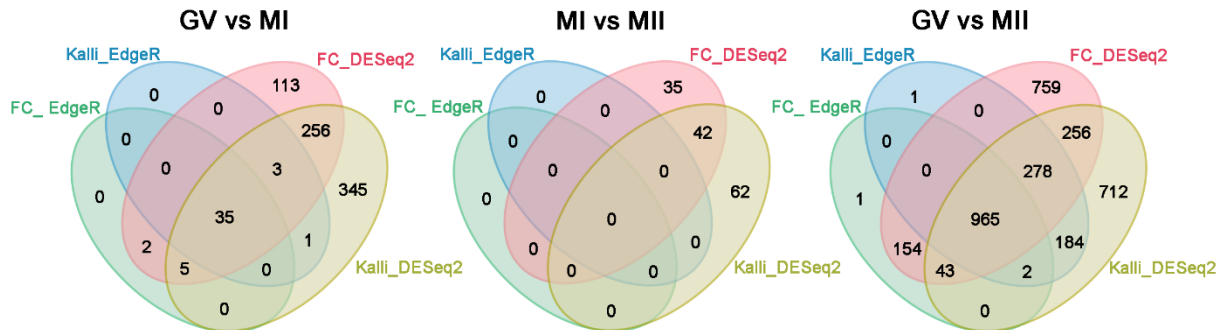


Figure 8 Venn Diagrams showing the number of DEGs obtained combining the different bioinformatic tools utilized.

Among the 42 DEGs identified between GV and MI stages, 38 were upregulated in MI oocytes, while 4 were downregulated (Fig. 9A). Gene Ontology (GO) analysis of the upregulated genes in MI revealed significant enrichment for terms related to the “mitotic cell cycle”(Table 8).

Table 8 Gene Ontology analysis of Differentially Expressed Genes obtained in the MI -GV comparison.

MI vs GV			
Downregulated in MI			
GO	Term	N° of genes	p-value
MF	Atg12 activating enzyme activity	1	0.037
MF	Atg8 activating enzyme activity	1	0.037
Upregulated in MI			
GO	Term	N° of genes	p-value
BP	mitotic cell cycle	10	0.003
REAC	Aberrant regulation of mitotic cell cycle due to RB1 defects	3	0.024
REAC	Diseases of mitotic cell cycle	3	0.028

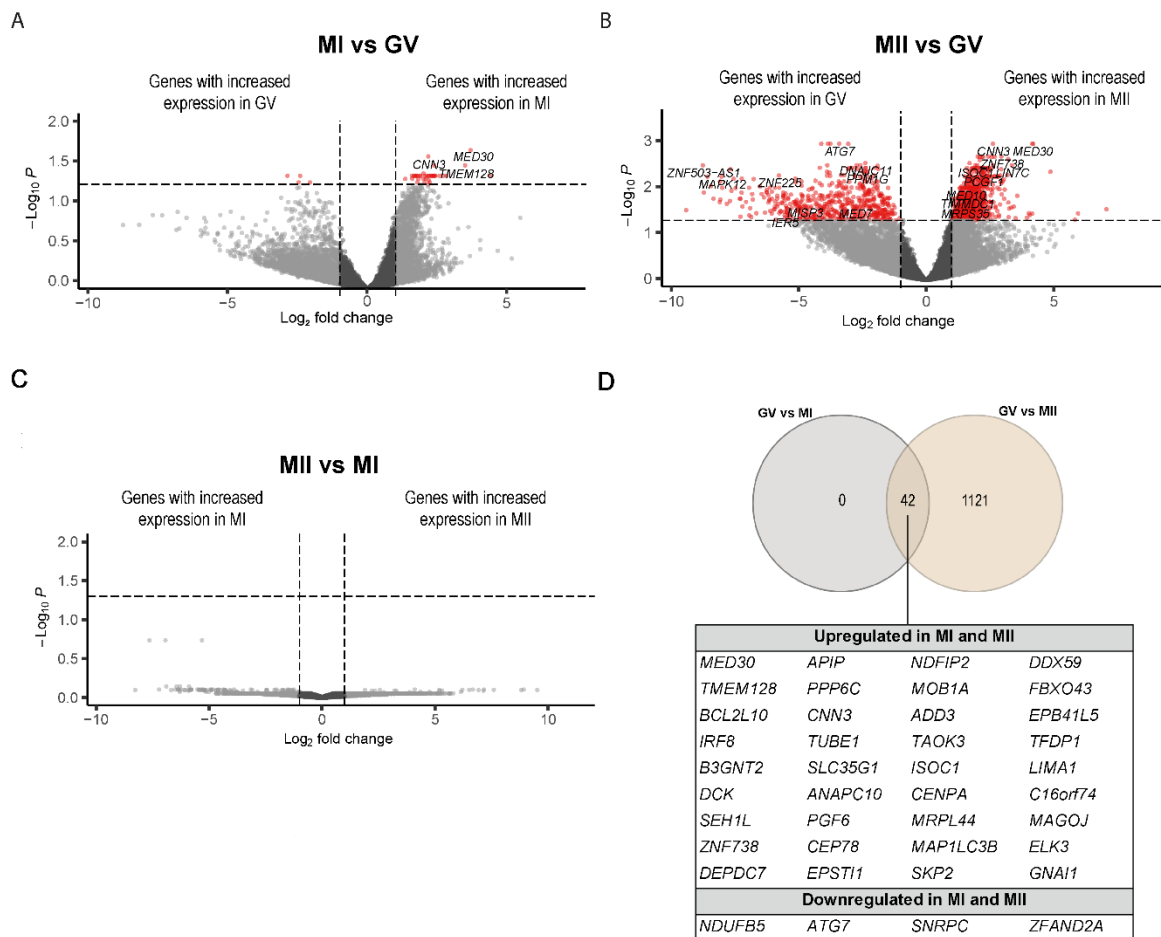


Figure 9 Differentially Expressed Genes during oocyte maturation. DEGs were defined as $FDR \leq 0.05$ and $FoldChange (FC) > |2|$. Volcano Plots for comparison between A) MI and GV oocytes B) MII and GV oocytes C) MII and MI oocytes. D) Venn diagram of DEGs obtained from the comparisons between MI vs. GV and MII vs. GV, showing the overlap and specific DEGs. GV – Germinal-Vesicle; MI – Metaphase I; MII – Metaphase II.

In the comparison between GV and MII oocytes, a total of 1165 DEGs were detected, with 635 upregulated and 528 downregulated in MII oocytes (Fig. 9B). GO analysis indicated that MII-upregulated genes were primarily associated with cell cycle regulation, RNA localization and transport, chromosome organization, and segregation (Fig. 10). In contrast, downregulated genes were enriched for terms related to nucleoplasm and proteasome activity. Noteworthy, all the 42 DEGs identified in the GV vs MI comparison were also present in the GV vs MII comparison, whereas 1121 DEGs were unique to the GV vs MII comparison (Fig. 9D).

Interestingly, no DEGs were identified between MI and MII oocytes, reflecting a similar transcriptional state between oocytes in these two maturation stages (Fig.9C).

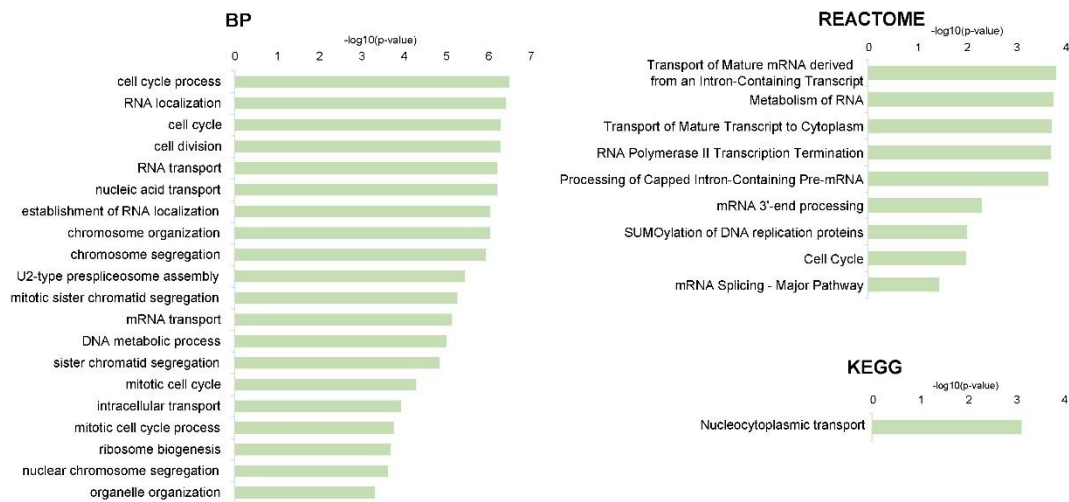


Figure 10 Functional enrichment analysis, displaying the significant Gene Ontology (GO) biological processes (BP), REACTOME pathways, and KEGG pathways associated with upregulated DEGs in MII relative to GV oocytes.

Expression plots of several DEGs are presented in Fig. 11, highlighting key genes involved in oocyte maturation. Among these, *WEE2* and *DPPA3*, two of the top 10 highly expressed genes (Table 6), exhibited consistent upregulation across all maturation stages. *WEE2* encodes a kinase that plays a pivotal role in regulating meiosis, phosphorylates and inhibits CDK1/CDC2 (Sang, Li et al. 2018). *DPPA3* (Developmental Pluripotency Associated 3), also called STELLA, plays an important role in shaping the oocyte methylome, preventing de novo methylation by DNMT1 (Li, Zhang et al. 2018)).

Another prominently upregulated gene is *CDC20* (*cell division cycle 20*), which encodes a protein functioning as a co-activator subunit of the anaphase-promoting complex/cyclosome (APC/C). In oocytes, this protein is important for the activation of APC/C, ensuring accurate chromosome segregation and promoting the exit of meiosis I (Jin, Hamada et al. 2010).

In contrast, several genes showed significant downregulation during the different stage of maturation. For instance, *PANX1* and *PATL2* are two notable downregulated genes with known implications in oocyte development. *PANX1* encodes pannexin 1, a glycoprotein that has an important role in cellular communication, namely for the transport of adenosine 5'-triphosphate (ATP). Variants in *PANX1* may lead to aberrant ATP release followed by oocyte death (Wang and Pepling 2021). Similarly, *PATL2* encodes an RNA-binding protein involved in translational repression, which is critical for maintaining the meiotic arrest of oocytes. Biallelic mutations in *PATL2* have been associated with oocyte GV arrest and infertility, highlighting its essential role in early stages of oocyte development (Chen, Zhang et al. 2017).

Another important gene exhibiting downregulation throughout maturation is *ZAR1* (*Zygote Arrest 1*). This gene is involved in coordinating maternal mRNA storage, translation, and degradation, processes essential for early embryonic development (Rong, Ji et al. 2019).

MED30, *ZFAND2A*, and *ZNF738* stand out among the most statistically significant DEGs due to their distinct expression patterns. *MED30*, Mediator Complex Subunit 30, and *ZNF738*, a zinc finger protein, both showed upregulation in MII oocytes.

In contrast, *ZFAND2A*, *Zinc Finger AN1-Type Containing 2A*, was markedly downregulated in MII oocytes.

Together, these findings provide a comprehensive view of the dynamic transcriptional changes occurring during oocyte maturation, emphasizing the interplay between genes that drive meiotic progression and genes that signal the end of earlier developmental roles.

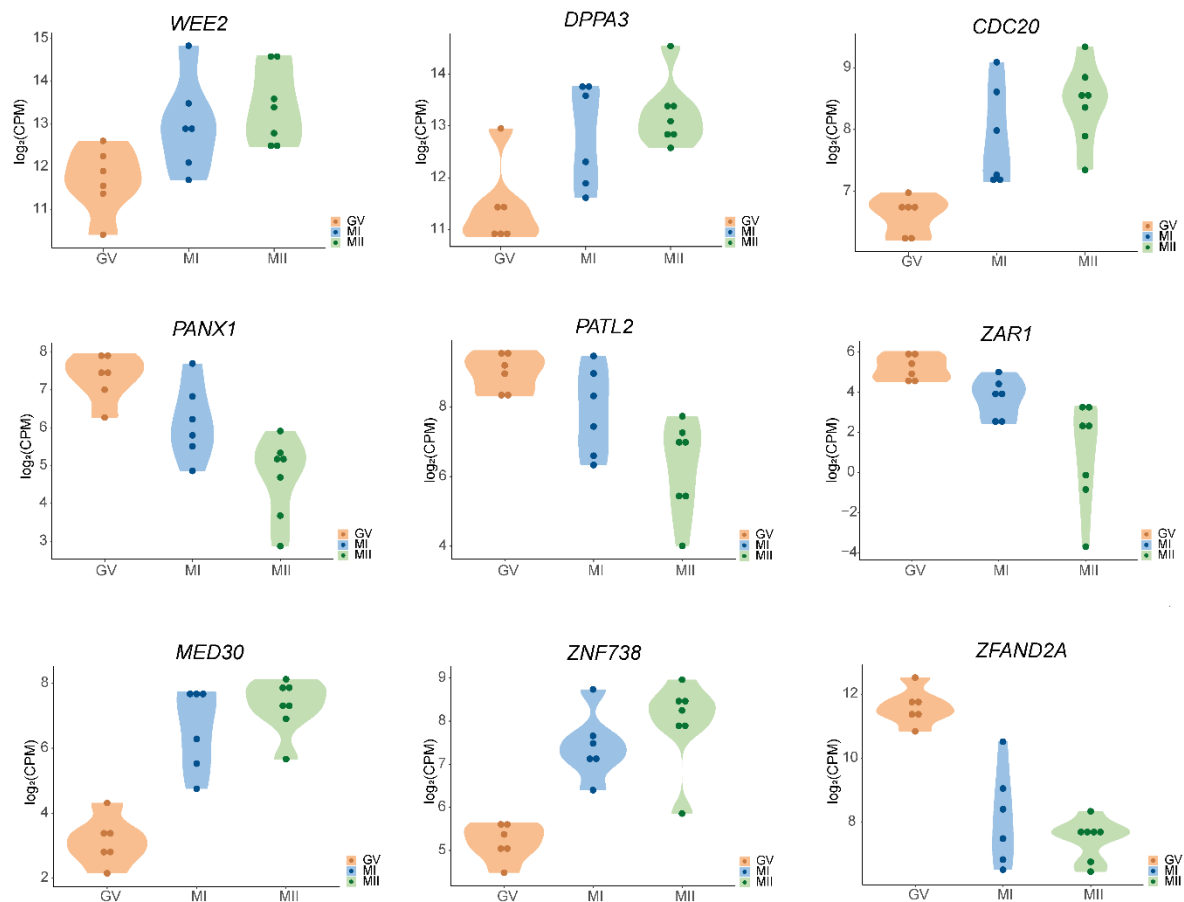


Figure 11 Violin plots of Differentially Expressed Genes (DEGs) during oocyte maturation. Violin plots illustrate the expression levels of selected DEGs with FDR ≤ 0.05 and absolute Fold Change (FC) $> |2|$, specifically for the genes: *WEE2*, *DPPA3*, *CDC20*, *PANX1*, *PATL2*, *ZAR1*, *MED30*, *ZNF738* and *ZFAND2A*. Orange: GV oocytes, Blue: MI oocytes, Green: MII oocytes.

1.4. Expression of epigenetic regulators throughout human oocyte maturation

Epigenetic modifications are fundamental for regulating oocyte meiotic maturation, orchestrating the transcriptional and structural changes necessary for developmental progression. To investigate the epigenetic landscape during this process, we analyzed the expression of several key epigenetic regulators (Fig. 12).

Among the HDACs, *HDAC9*, *HDAC2*, and *HDAC3* exhibited consistently high expression across all stages of oocyte maturation (Fig. 12A). Additionally, SIRT, a class of nicotinamide adenine dinucleotide (NAD⁺)-dependent histone deacetylases (Wu, Zhang et al. 2022), also displayed notable expression patterns. While *SIRT7* and *SIRT5* were highly expressed throughout maturation, *SIRT3* and *SIRT6* showed differential expression, with significant downregulation in MII oocytes (Fig. 12B).

Histone acetyltransferases (HATs) also demonstrated prominent roles during oocyte maturation. Among these, *HAT1* and *KAT7* were the most highly expressed throughout meiotic maturation (Fig. 12C).

Histone demethylases (KDMs), particularly *KDM1B* and *KDM3A*, were consistently expressed across all maturation stages (Fig. 12D). Of particular note, *KDM3AP1* was identified as a differentially expressed gene, with increased expression in MII oocytes.

We also examined the expression of histone methyltransferases, including SET domain-containing proteins (SETDs) and lysine methyltransferases (KMTs). Among the SETD family, several members exhibited stable expression (Fig. 12E). However, the *KMT2C* gene stood out as the most highly expressed methyltransferase across all stages (Fig. 12F). Additionally, of all the histone methyltransferases, only *KMT2E* was identified as a DEG, showing upregulation in MII oocytes.

DNA methylation, another critical epigenetic mechanism, also revealed dynamic regulation during oocyte maturation. Among the DNA methylation writers, *DNMT1* (*DNA methyltransferase 1*) was highly expressed across all stages and showed upregulation in MII oocytes. This upregulation was accompanied by increased expression of *UHRF1*, a known DNMT1 recruiter, and *DPPA3*, which binds to methylated DNA (Fig. 12G). These findings may suggest an active role for DNMT1 and its associated factors in maintaining or modifying DNA methylation patterns during oocyte maturation. Regarding *DNMT3A* and *DNMT3B*, these genes had a lower expression than DNMT1 and the expression of these methyltransferases remained constant throughout the stages of maturation. In contrast, *DNMT3L* was not expressed at any stages of maturation.

As for DNA demethylation processes, members of the TET family of enzymes were analyzed. *TET3* emerged as the most highly expressed demethylase across all maturation

stages, consistent with its known role in the conversion of 5mC to 5hmC during the early zygote stage (Wossidlo, Nakamura et al. 2011). The high expression of *TET3* supports its crucial function in preparing the oocyte genome for subsequent embryonic development. In terms of imprinted genes, several key transcripts displayed high expression levels across all maturation stages, including *MEST* (*Mesoderm-Specific Transcript*), *UBE3A* (*Ubiquitin Protein Ligase E3A*), and *NLRP2* (*NLR Family Pyrin Domain Containing 2*) (Fig. 12H). Notably, *MEST* was upregulated in MII oocytes, while *RASGRF1* (*Ras Protein Specific Guanine Nucleotide Releasing Factor 1*) was downregulated during this stage. In summary, our analysis highlights the dynamic and tightly regulated expression of epigenetic modulators during oocyte maturation. These regulators, spanning histone modifiers, DNA methylation machinery, and imprinted genes, underscore the complex interplay of epigenetic mechanisms essential for the successful transition of oocytes through meiotic maturation.

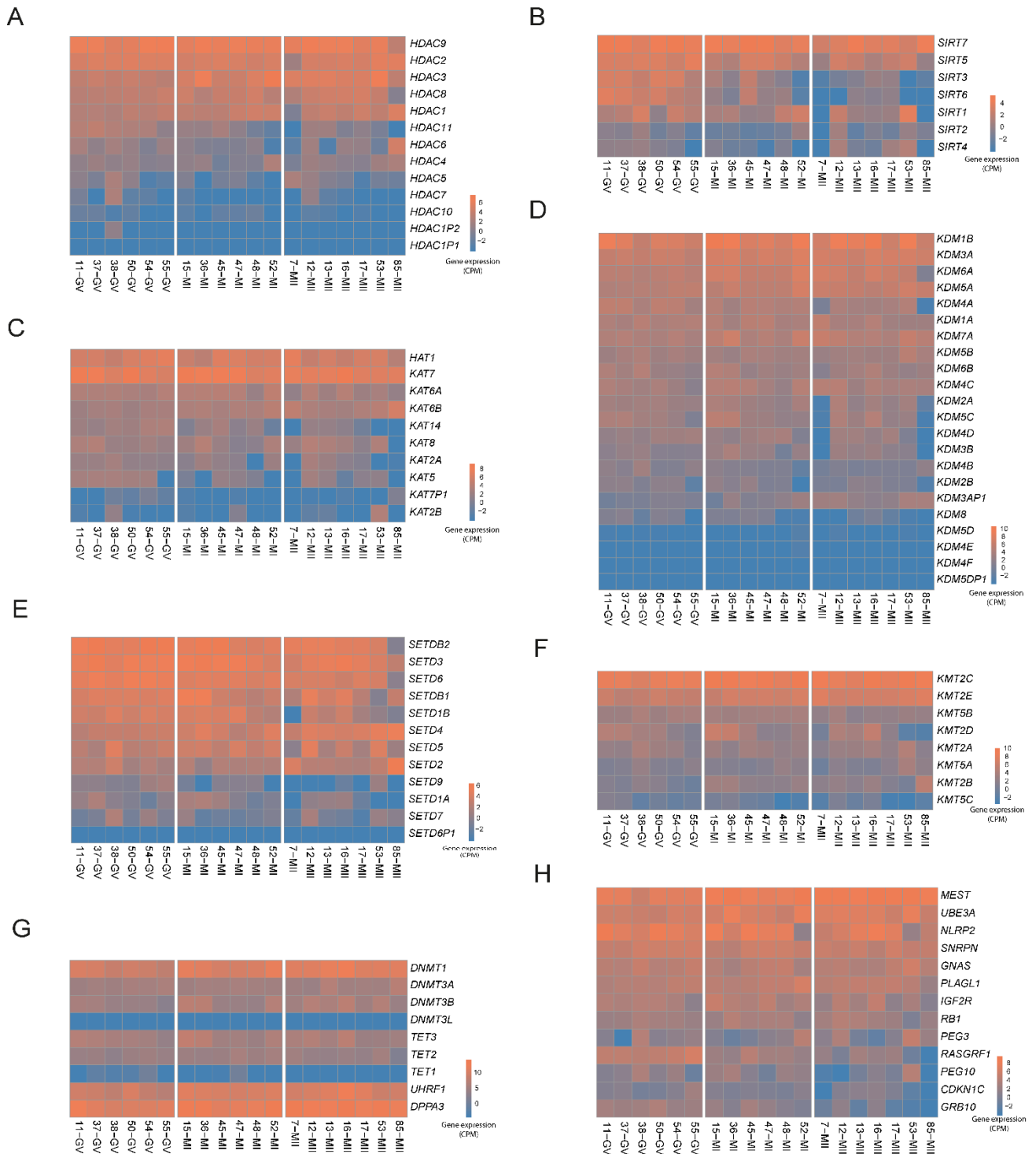


Figure 12 Heatmaps of epigenetic regulators and imprinted genes. Histone deacetylases (A) and Sirtuins (B); Histone acetyltransferases (C); Histone demethylases (D); SET-domain protein methyltransferases (E); Histone Methyltransferases (F); DNA methyltransferases and co-factors and Demethylases (G); Imprinted genes (H). The color gradient represents the level of gene expression (in CPM).

2. Confirmation of *ZFAND2A* and *DNMT1* expression by quantitative RT-PCR

The expression patterns of *ZFAND2A* and *DNMT1* were independently validated using qRT-PCR in an additional set of samples. These findings further confirm their significant roles in regulating key processes during oocyte maturation (Fig. 13).

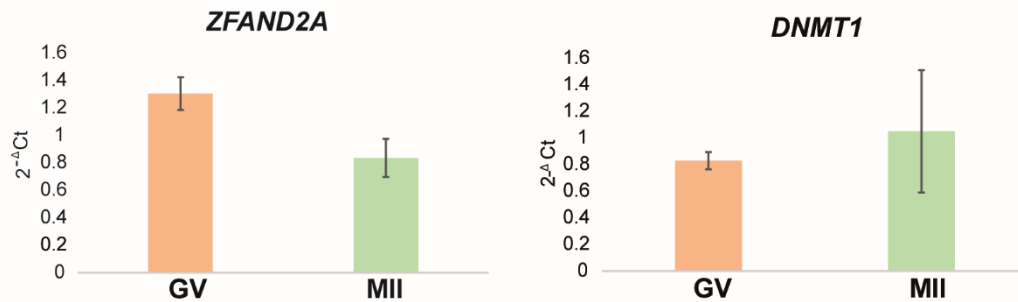


Figure 13 qRT-PCR confirmation of differential gene expression for *ZFAND2A* and *DNMT1* genes. An independent set of human oocytes was used for qRT-PCR confirmation, 10 GV oocytes and 3 MI oocytes for *ZFAND2A* and 9 GV oocytes and 4 MI oocytes for *DNMT1*.

3. Immunofluorescence profiling in oocytes from different maturation stages

To complement the transcriptomic findings, immunofluorescence analyses were performed for TET3 and *DNMT1*, two key epigenetic regulators that exhibited high expression levels across oocyte maturation stages. These proteins play critical roles in DNA methylation dynamics, contributing to essential processes of epigenetic reprogramming.

Initially, actin staining was performed as a structural control to assess oocyte integrity and ensure the accuracy and reliability of the immunofluorescence technique. Actin was shown to be enriched in the cytoplasm and in the cortex, as expected (Fig.14).

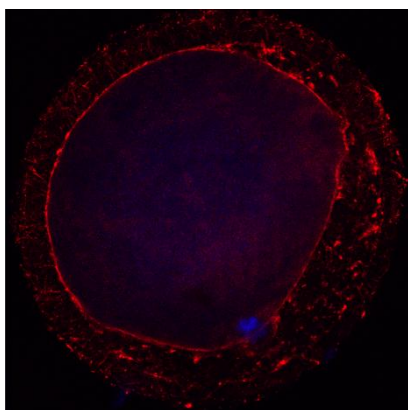


Figure 14 Immunofluorescence staining of MI oocyte. Actin was stained in red and nucleus in blue.

Immunofluorescence was utilized to assess the localization and intensity of TET3 and DNMT1, the enzyme responsible for maintaining DNA methylation patterns.

DNMT1, the primary DNA methyltransferase responsible for maintaining DNA methylation patterns during replication, showed a cytoplasmic localization in GV and MI oocytes, although the intensity was very low (Fig.15).

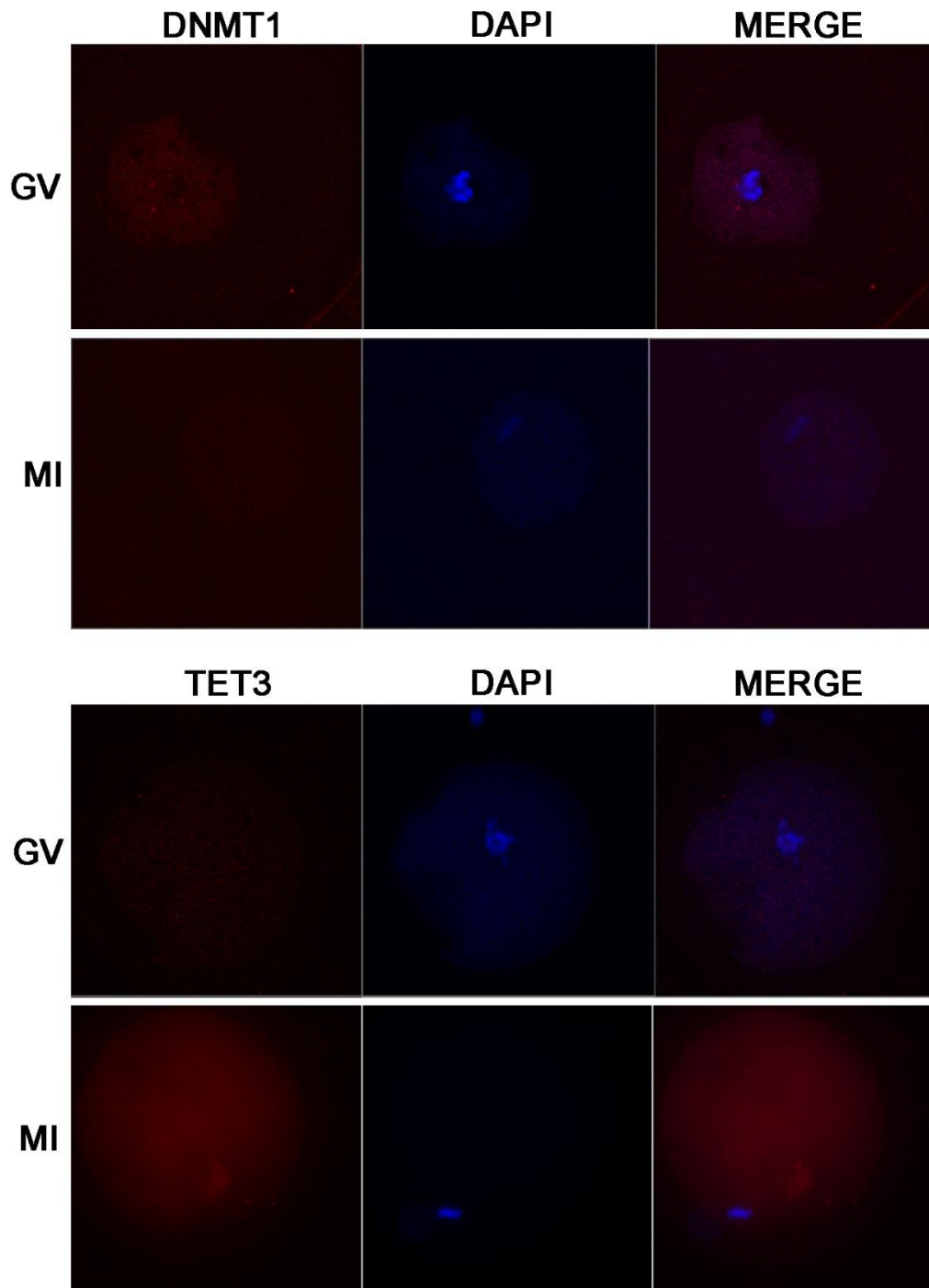


Figure 15 Immunofluorescence staining of immature oocytes in GV and MI stages. DNMT1 was stained in red and nucleus in blue.

Similarly, TET3, an enzyme involved in active DNA demethylation through the conversion of 5mC to 5hmC, also showed cytoplasmic localization (Fig.15).

Regarding 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), we detected their presence consistently across all stages of oocyte maturation. Both epigenetic modifications were predominantly localized within the nucleus, underscoring their potential role in regulating transcriptional dynamics during meiotic progression. Notably, a distinct alteration was observed in MII oocytes. At this stage, 5mC was identified not only in the nucleus but also in the cortical region and the polar body. Similarly, 5hmC was localized within the nucleus and prominently in the polar body (Fig.16).

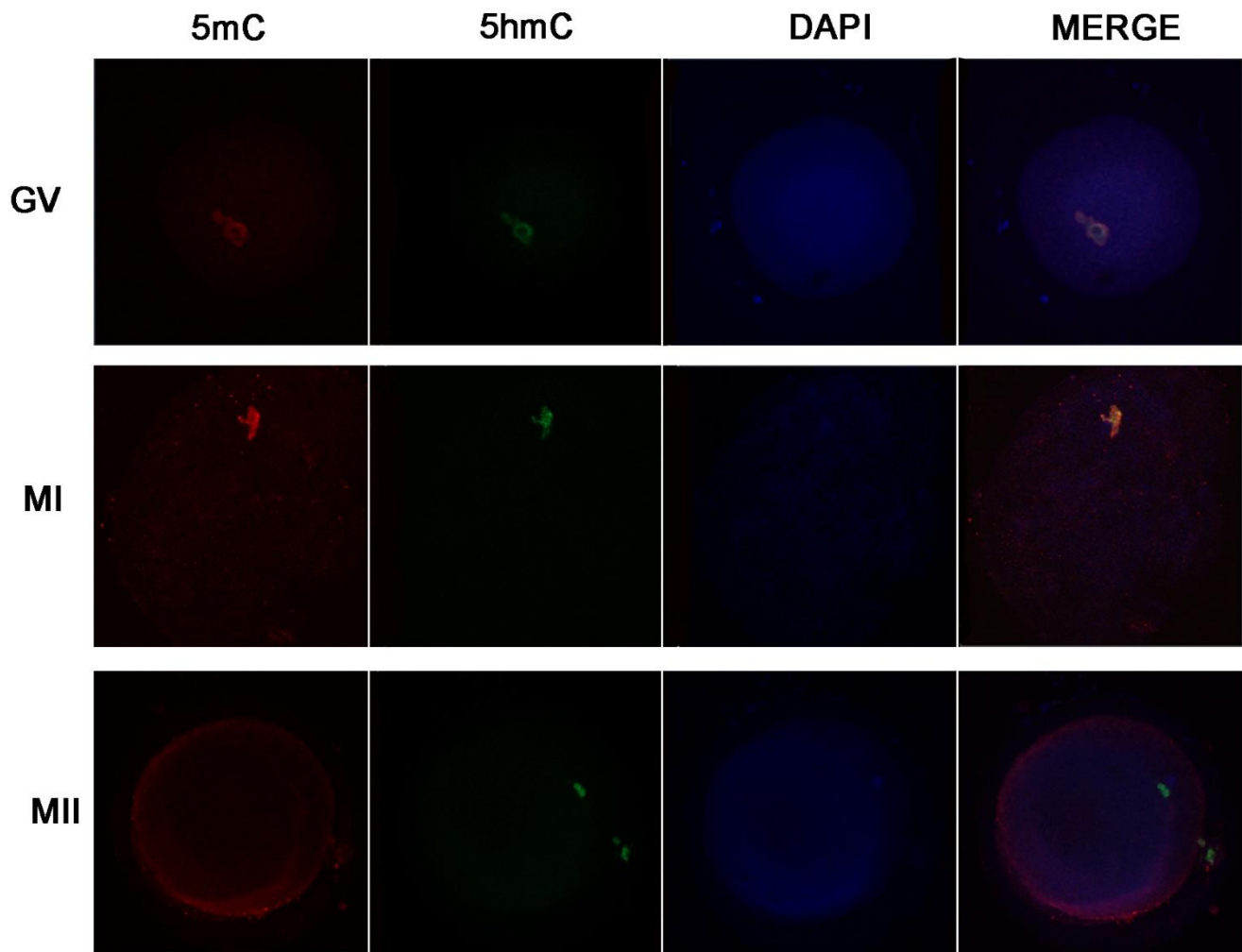


Figure 16 Immunofluorescence staining of oocytes in GV, MI and MII stages. 5 methylcytosine was stained in red, 5 hydroxymethylcytosine in green and nucleus in blue.

DISCUSSION

In this study, we conducted a comprehensive transcriptomic analysis of human oocytes at three key developmental stages of the meiotic maturation process: Germinal Vesicle (GV) stage, corresponding to prophase I; the Metaphase I (MI) stage, during the first meiotic division; and the Metaphase II (MII) stage, during the second meiotic division. By profiling the transcriptome at these critical points, we aimed to deepen our understanding of the molecular mechanisms driving oocyte maturation and to identify potential gene expression patterns specific to each stage.

An optimal oocyte morphology is characterized by a spherical structure enclosed within a uniform zona pellucida, a homogeneous and translucent cytoplasm free of inclusions, and a properly sized polar body (Medicine and Embryology 2011). Currently, the quality of oocytes in clinical practice is predominantly assessed using morphological markers, such as cytoplasmic appearance, polar body morphology, oocyte size, zona pellucida thickness and perivitelline space. However, these traditional markers provide limited insight into the molecular and functional state of the oocytes

Recent studies have highlighted the critical need for molecular markers to improve the precision of oocyte quality assessments and predict their developmental competence (Sang, Zhou et al. 2021).

Our work addresses this gap by focusing on the transcriptomic signatures of human oocytes, which may serve as valuable molecular indicators of oocyte quality and maturation potential. This knowledge could enable the future development of innovative diagnostic tools and strategies to improve assisted reproductive technologies (ART).

Our findings illustrate the dynamic changes in gene expression patterns during human oocyte maturation, highlighting a reduction in the number of expressed genes across developmental stages—from the Germinal Vesicle (GV) stage, through Metaphase I (MI), and subsequently to Metaphase II (MII). This observed decline supports the well-documented RNA degradation accompanying oocyte maturation, a phenomenon critical for the preparation of oocytes for successful fertilization and early embryonic development (Jiang and Fan 2022). In fact, disruptions in mRNA degradation during late oogenesis and early embryogenesis can result in developmental abnormalities and are linked to various human diseases (Yang, Xin et al. 2024).

This trend has been corroborated by several studies, which emphasize the substantial decrease in transcript abundance during oocyte maturation (Yu, Doni Jayavelu et al. 2020, Llonch, Barragán et al. 2021, Yan, Gu et al. 2021, Takeuchi, Yamamoto et al. 2022). Indeed, in a recent meta-analysis including human oocyte transcriptomic data from 6 independent

studies, a 30% decline in total mRNA from GV to MII stage was described (Ducreux, Ferreux et al. 2023). Oocyte development is accompanied by two waves of maternal transcript degradation to prepare for the embryonic transcriptome: the maternal decay (M-decay) during oocyte maturation, which removes 30–50% of maternal factors, and the zygotic decay (Z-decay) after fertilization, which eliminates the remaining maternal material. This results in approximately 80% of polyadenylated mRNAs from the GV stage being absent in the eight-cell embryo (Ducreux, Ferreux et al. 2023).

These processes are regulated by RNA-binding proteins, microRNAs, and other decay factors. Maturation to the MII stage does not guarantee embryo viability; instead, oocyte competence relies on the precise degradation, conservation, and regulation of specific maternal transcripts at each developmental stage.

Therefore, a tightly regulated process of transcript clearance, which is crucial for eliminating maternal RNA that can prevent EGA and ensure the oocyte's suitability for subsequent developmental stages.

Interestingly, our findings reveal a decline in the number of unique genes—genes exclusively expressed at a specific oocyte stage—from GV to MII, corroborating observations by others (Yu, Doni Jayavelu et al. 2020), albeit the number of unique genes we observed is substantially higher than the number reported by Yu and collaborators. Notably, 72 genes were found to be uniquely expressed in the MII stage, while 635 genes were upregulated during oocyte maturation from GV to MII stages, highlighting the active role of transcriptional regulation across meiotic divisions.

As proposed by Ducreux and collaborators, this pattern may reflect the pool of mRNAs available for translation that have evaded degradation due to shorter polyA tails. Alternatively, it could be attributed to polyA tail elongation during the GV to MII transition, which enhances their stability and translation potential in MII oocytes (Ducreux, Ferreux et al. 2023). These findings underscore the intricate regulation of the transcriptome during oocyte maturation, emphasizing the interplay between mRNA stability, degradation, and translation.

We identified a substantial number of DEGs when comparing GV and MII oocytes - 1165 genes - including several that are critical for oocyte maturation. Notably, 148 of these DEGs overlapped with those reported by Pietroforte and collaborators (Pietroforte, Barragan Monasterio et al. 2023), underscoring the consistency of our findings with previous studies. Among the overlapping DEGs, key genes such as *ZAR1*, *PATL2*, *TRIP13*, *RBBP7*, and

WEE2 stand out due to their established roles in regulating oocyte maturation and meiotic progression.

Additionally, *ZAR1*, *PATL2*, *RBBP7*, and *WEE2* were also identified as differentially expressed genes by Yu and collaborators, between MII oocytes and MI oocytes (Yu, Doni Jayavelu et al. 2020), highlighting the robustness of the results across different laboratories and studied populations.

The maintained differential expression of these genes across oocyte maturation stages emphasizes their functional roles, including transcriptional regulation, chromosomal segregation, and cytoplasmic maturation. For instance, *ZAR1* is essential for oocyte-to-embryo transition by coordinating maternal mRNA storage, translation and degradation (Wu, Viveiros et al. 2003). *Zar1*-null (*Zar1*^{-/-}) mice are viable however *Zar1*^{-/-} females are infertile, with embryos arresting at the one-cell stage due to impaired transcription complex synthesis (Wu, Viveiros et al. 2003, Rong, Ji et al. 2019). A study in mice found that *ZAR1* protein levels decrease during oocyte development, including during maturation (Rong, Ji et al. 2019). This is consistent with the decline in gene expression noted in our study. *PATL2* is an oocyte-specific RNA-binding protein, and studies have confirmed that *PATL2* mutation in humans causes oocyte maturation arrest (Chen, Zhang et al. 2017, Christou-Kent, Kherraf et al. 2018). In fact, it has been shown that *PATL2* deletion caused the global decay of poly(A) mRNA in immature oocytes leading to female sterility. However, in MII oocytes, the deletion of *PATL2* does not have this impact. Therefore, *PATL2* stabilizes mRNA in growing oocytes by recruiting EIF4E and CPEB1. During oocyte maturation, *PATL2* is phosphorylated and degraded via ubiquitination, releasing EIF4E and CPEB1 to activate polyadenylation and translation (Zhang, Liu et al. 2023). The decrease in *PATL2* expression observed in our study during oocyte maturation may highlight the importance in an early stage of maturation

Contrarily, *WEE2* expression is higher in MII oocytes than in immature oocytes. However, it appears to be involved in both GV and MII maturation stages. In mouse oocytes, *Wee2* is crucial to maintaining meiotic arrest at the GV stage and enabling MII exit (Han, Chen et al. 2005, Oh, Susor et al. 2011). Its downregulation leads to high MPF activity, preventing MII exit and pronucleus formation, resulting in fertilization failure (Han and Conti 2006). In human oocytes, variations in *WEE2* were associated with human pronucleus formation and fertilization failure (Zhang, Mu et al. 2019). At the protein level, *WEE2* was also observed to be more enriched in the MII oocyte, consistent with the increase in gene expression observed in our study (Virant-Klun, Leicht et al. 2016).

Among the genes analyzed, *TUBB8* emerged as the most highly expressed across all three maturation stages. *TUBB8* encodes a major beta-tubulin subunit that is crucial in oocytes and early embryogenesis, playing a pivotal role in meiotic spindle assembly and the maturation of human oocytes. Mutations in *TUBB8* can lead to maturation arrest at meiosis I, primarily due to disrupted microtubule behaviour and defective oocyte spindle assembly, ultimately causing female infertility (Feng, Sang et al. 2016).

In addition to *TUBB8*, its paralog *TUBB8B* and the pseudogene *TUBB8P7* were also highly expressed across all three stages. While *TUBB8B* is a protein-coding gene sharing structural and functional similarities with *TUBB8*, *TUBB8P7* represents a pseudogene with no coding capacity. *TUBB8P7* was observed to be degraded during progression to the blastocyst stage, specifically between the 4-cell and 8-cell stage (Bouckenheimer, Fauque et al. 2018).

Several mitochondrial genes exhibited high expression levels across all stages of oocyte maturation, supporting the well-documented abundance and critical role of mitochondria in oocytes. Indeed, oocytes contain thousands of mitochondria, which originate from the few hundred present in PGCs; however, the major increase in mitochondrial number occurs during the later stages of folliculogenesis. Oocyte quality correlates with both the amount of mtDNA and the amount of ATP, produced by mitochondria. mtDNA copies surge at two key stages: the transition from primordial to primary follicles and the transition from GV to MII oocytes in late antral follicles (Kirillova, Smitz et al. 2021).

Among the genes with high expression, *MT-ND4* (encoding a subunit of NADH dehydrogenase) and *MT-CO1* (encoding a subunit of cytochrome c oxidase) stood out as the most highly expressed. These genes are integral components of the mitochondrial electron transport chain, which is essential for ATP production. Therefore, their elevated expression underscores the high energy demands of oocyte maturation and early embryonic development and may also serve as biomarkers for mitochondrial activity and oocyte competence.

Another highly expressed gene was the *H3-3B* that codes for histone variant H3.3, which was shown to be essential for paternal chromatin remodelling after fertilization and for the subsequent paternal genome activation during embryogenesis (Kong, Banaszynski et al. 2018).

Other noteworthy genes include *DPPA3* (also known as STELLA), essential for female fertility. *DPPA3* encodes a protein that plays a pivotal role in shaping the oocyte methylome,

preventing *de novo* methylation through the inhibition of UHRF1-mediated DNMT1 activity, thus maintaining the epigenetic integrity of the oocyte (Li, Zhang et al. 2018).

Furthermore, another gene indispensable for meiosis and oocyte maturation, *OOSP2*, was also highly expressed during maturation. Hu and colleagues recently proposed that *OOSP2* promotes oocyte maturation by activating the small GTPase signalling pathway. This activation triggers a cascade of molecular and cellular events that propagate from the cytoplasm to the nucleus, orchestrating the progression of maturation processes. Additionally, they observed that *OOSP2* can upregulate the protein levels of some genes without increasing their transcriptional expression, indicating a post-transcriptional regulation and translational of gene expression (Hu, Zeng et al. 2022). Therefore, our study confirms and complements previously reported data, highlighting the relevance of *OOSP2* as a multifaceted regulator in oocyte maturation and development.

Two other highly expressed genes, *BMP15* (*Bone morphogenetic protein 15*) and *GDF9* (*Growth differentiation factor 9*), encode oocyte-derived growth factors that regulate granulosa cell proliferation and differentiation. These factors also promote antrum formation, highlighting their essential roles in folliculogenesis. Through paracrine signalling, these factors promote mitosis and differentiation of follicular somatic cells, facilitating follicular development (Alam and Miyano 2020). Additionally, *GDF9* and *BMP15* mRNA levels in cumulus cells correlate with oocyte maturation, embryo quality, and pregnancy outcomes, suggesting their potential as markers of oocyte developmental competence. (Li, Li et al. 2014). In our study, the high expression of *BMP15* and *GDF9* across various stages of maturation underscores their critical roles in oocyte-somatic cell communication and folliculogenesis.

Regarding the differential gene expression observed throughout oocyte maturation, our analysis revealed distinct patterns of regulation. Genes associated with the cell cycle, RNA processing, and epigenetic regulation were significantly upregulated in MII oocytes, consistent with the findings of Ducreux and collaborators (Ducreux, Ferreux et al. 2023). This upregulation highlights the increased transcriptional and epigenetic activity required to prepare the oocyte for fertilization and subsequent embryonic development.

Conversely, our study identified that genes related to the proteasome complex were downregulated in MII oocytes. This pattern suggests that maternal protein degradation predominantly occurs before the MII stage. During the MII oocyte arrest, proteasome activity appears to be inhibited or halted, aligning with observations reviewed by (Karabinova, Kubelka et al. 2011). The decrease in protein degradation at this stage may serve to

preserve some maternal proteome, ensuring stability during the critical period of fertilization and early embryonic development.

Epigenetic regulation plays a crucial role in maintaining genome stability and ensuring proper developmental potential during oocyte maturation. As mentioned before, genes associated with epigenetic regulation were increased in MII oocytes in our study. One of these genes is *DNMT1*, which is considered a maintenance DNA methyltransferase, i.e., faithfully copies methylation patterns during DNA replication in the S-phase of the cell cycle. Apart from being a DEG, this methyltransferase was also found to be highly expressed throughout oocyte maturation, as observed by others (Yu, Doni Jayavelu et al. 2020). Our group's previous research also observed high expression of *DNMT1* during meiotic divisions of human spermatogenic cells (Marques, João Pinho et al. 2011), suggesting that DNMT1 might play a role in methylation repair to prevent methylation errors, particularly at imprinted genes, to be transmitted to the next generation.

Indeed, early studies posited that DNMT1 could act as a *de novo* methyltransferase (Yoder, Soman et al. 1997), and subsequent work demonstrated that its activity is regulated by *DPPA3*, which safeguards the unique epigenome of the oocyte (Li, Zhang et al. 2018). Additionally, UHRF1 (also known as NP95), a key protein believed to recruit DNMT1 to hemi-methylated DNA during replication (Sharif, Muto et al. 2007), also showed high expression levels across all stages of oocyte maturation. The co-expression of DNMT1 and UHRF1 highlights the tightly regulated mechanisms that maintain epigenetic integrity in oocytes.

These findings collectively suggest that DNMT1, possibly in collaboration with UHRF1, not only preserves methylation patterns but also plays a crucial role in maintaining epigenetic stability during oocyte development. This dual role is essential for safeguarding the maternal epigenome and ensuring proper developmental potential in the next generation. Further studies are warranted to elucidate the specific regulatory mechanisms and functional interplay of these factors during gametogenesis.

To verify the presence of this epigenetic regulator, we performed immunofluorescence for DNMT1 in human oocytes. We observed cytoplasmic staining in both GV and MI oocytes. Petrusa and colleagues observed that DNMT1 was present throughout maturation but shifted from the nucleus (GV) to the cytoplasm (MI, MII), supporting methylation maintenance (Petrussa, Van de Velde et al. 2014). Regarding our study, our technique had some limitations and would need to be performed on a larger number of oocytes to increase the confidence in the obtained results.

DNMT3A and *DNMT3B* are involved in de novo methylation, with *DNMT3B* expression increasing in fully-grown oocytes (Okoe, Chiba et al. 2014, Yan, Gu et al. 2021). *DNMT3A* and *DNMT3B* are present in the oocyte cytoplasm of all stages of maturation but not in the nucleus (Petrussa, Van de Velde et al. 2016). In our study, *DNMT3A* and *DNMT3B* expression remains constant during oocyte maturation and the level of expression was lower than *DNMT1*. Notably, *DNMT3L* was not expressed at any stage of oocyte maturation, aligning with findings from previous studies (Okoe, Chiba et al. 2014).

Regarding DNA demethylation, we observed that two putative DNA demethylases, *TET2* and *TET3*, were expressed across all stages of oocyte maturation. Among these, *TET3* has been previously shown in mouse zygotes to play a critical role in the active DNA demethylation of the paternal pronucleus, facilitating the erasure of epigenetic marks and enabling subsequent zygotic development (Gu, Guo et al. 2011, Wossidlo, Nakamura et al. 2011). Our findings confirm the expression of these key enzymes during human oocyte maturation, suggesting their potential involvement in maintaining the epigenetic landscape required for successful fertilization and early embryonic development. However, we observe that *TET3* signal in GV and MI oocytes was very low and was localized in the cytoplasm.

Another additional analysis performed on oocytes at the three stages of maturation was the immunofluorescence assessment of the epigenetic modifications 5-methylcytosine and 5-hydroxymethylcytosine. We observed the presence of both epigenetic modifications, 5mC and 5hmC, across all stages of oocyte maturation, predominantly localized in the nucleus. However, in MII oocytes, 5hmC was also detected in the polar body. Regarding 5mC, cortical staining was observed, which may suggest incomplete antibody penetration; nonetheless, nuclear staining of this marker was also present. Other studies have also detected 5mC and 5hmC in oocytes in different maturation stages. Studies using single-cell whole-genome bisulfite sequencing (SC-WGBS) have shown that CpG methylation levels remain stable (48–53.8%) across maturation stages (GV, MI, and MII) (Guo, Zhu et al. 2014, Okoe, Chiba et al. 2014, Yu, Dong et al. 2017, Ye, Yang et al. 2020). In contrast, non-CpG methylation (CHG and CHH) increases during oocyte maturation and correlates with gene expression changes, likely mediated by *DNMT3B* (Yu, Dong et al. 2017, Yu, Doni Jayavelu et al. 2020). Non-CpG methylation is enriched in gene bodies and intergenic regions, suggesting it plays a role in transcriptional regulation during maturation (Guo, Zhu et al. 2014, Ye, Yang et al. 2020).

In the case of 5hmC, previous studies in human oocytes have reported nuclear localization throughout maturation, with a slight decrease in signal intensity as oocytes progress through

their developmental stages (De Munck, Petrusa et al. 2015, Petrusa, Van de Velde et al. 2016). This work is among the few studies to investigate 5mC and 5hmC in human oocytes across all three stages of maturation. Yan and colleagues (Yan, Gu et al. 2021) specifically analyzed MII oocytes and found that genome-wide 5hmC levels in these oocytes were approximately 1.60%. Among various genomic elements, CpG islands (CGIs) exhibited the highest levels of DNA hydroxymethylation, whereas gDMRs, gene bodies, promoters, enhancers, intergenic regions, and repetitive elements showed relatively lower levels of 5hmC. Furthermore, Tet-dependent hyper-hydroxymethylated differentially methylated regions (hmDMRs) in the maternal pronucleus displayed a 5hmC percentage of 3.70% in MII oocytes.

This evidence highlights the dynamic nature of DNA methylation and hydroxymethylation during oocyte maturation, emphasizing their potential roles in transcriptional regulation and developmental competence.

Our study has several key strengths, beginning with using transcriptomic analysis on single oocytes. Single-cell RNA sequencing enables the understanding of gene expression patterns, uncovering differences that might be masked in bulk analyses (Wu, Zhang et al. 2022). This approach allows for a detailed examination of individual oocytes, providing a higher resolution of the molecular processes that occur during maturation. Another important strength is our focus on oocytes that underwent *in vivo* maturation, ensuring that our findings reflect physiological conditions and minimizing potential artefacts associated with *in vitro* culture systems.

Despite these strengths, certain limitations should be recognized. One potential weakness is the use of an oligo-dT primer for RNA capture and cDNA synthesis (Macaulay, Teng et al. 2016). This approach targets poly-A-tailed mRNAs, potentially excluding non-polyadenylated transcripts from the analysis. However, it is important to note that RNA-Seq protocols focusing exclusively on polyadenylated mRNAs are widely accepted and have been utilized in numerous studies analyzing the human oocyte transcriptome (Reyes, Silva et al. 2017, Yu, Doni Jayavelu et al. 2020, Llonch, Barragán et al. 2021, Ntostis, Iles et al. 2021, Takeuchi, Yamamoto et al. 2022).

Furthermore, it is well established that oocytes are transcriptionally silent during certain stages of maturation. As such, changes in gene expression are likely controlled by post-transcriptional mechanisms, such as cytoplasmic polyadenylation of stored mRNAs. The length and regulation of poly(A) tails play a critical role in translation efficiency and mRNA stability. During oocyte maturation, poly(A) tail lengths are dynamically regulated, with

important meiotic proteins displaying increased polyadenylation to support their translation during this period (Yang, Wang et al. 2020, Liu, Zhao et al. 2023).

In conclusion, our study not only provides new insights but also corroborates previous findings regarding gene expression dynamics in human oocytes at different stages of maturation. These results contribute to a deeper understanding of oocyte biology, offering valuable implications for improving oocyte quality assessment and optimizing fertility treatments. By elucidating the molecular mechanisms underpinning oocyte maturation, this research lays the groundwork for future studies aimed at advancing reproductive medicine.

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APPENDIX



Transcriptomic analysis and epigenetic regulators in human oocytes at



different stages of oocyte meiotic maturation

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ABSTRACT

Human oocytes are highly specialized cells with the capacity to store and regulate mRNAs during oocyte maturation, in preparation for post-fertilization steps.

Here we performed single-oocyte transcriptomic analysis of human oocytes in three meiotic maturation stages – Germinal Vesicle (GV; n = 6), Metaphase I (MI; n = 6) and Metaphase II (MII; n = 7).

Single-oocyte transcriptomic analysis revealed that the total number of expressed genes progressively decreased from GV to MII stages, with 9660 genes being transcribed in GV, 8734 in MI and 5889 in MII. The same tendency was observed for the number of uniquely expressed genes, with 1328 uniquely expressed genes in GV, 401 in MI and 72 in MII. GO analysis of the uniquely expressed genes showed distinct terms in GV oocytes such as transferase activity, organonitrogen compound metabolic process and ncRNA processing. Analysis of Differentially Expressed Genes (DEGs) between the three maturation stages revealed 1165 DEGs between GV and MII oocytes, with 635 being upregulated and 528 downregulated, 42 DEGs between GV and MI, with 38 being upregulated and 4 downregulated, and no significant changes in gene expression between MI and MII oocytes. Comprehensive analysis of epigenetic regulators showed high expression of several histone-modifying enzymes, namely deacetylases, acetylases, lysine demethylases and methyltransferases, and DNA methylation regulators, namely the maintenance methyltransferase *DNMT1* and its co-regulators *DPPA3* and *UHRF1*. Some of these epigenetic regulators were differentially expressed between maturation stages, namely *SIRT3*, *SIRT6*, *KDM3A1*, *KMT2E*, *DNMT1*, *DPPA3* and the *MEST* and *RASGRF1* imprinted genes.

Our study contributes with important information on the transcriptional landscape of human oocytes in different stages of meiotic maturation, providing important insights into candidate biomarkers of human oocyte quality.

1. Introduction

Human oocytes undergo complex cellular and molecular changes that are essential for the success of fertilization and embryo

development. It has long been shown that these changes involve highly coordinated nuclear and cytoplasmic processes that take place during oocyte maturation (Eppig et al., 1996). Maturation of the oocyte involves three morphologically distinct phases - germinal vesicle (GV),

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containing an intact nuclear envelope; metaphase I (MI) showing a homogeneous cytoplasm; and metaphase II (MII), after extrusion of the first polar body, which remain arrested until fertilization occurs (Sang et al., 2021).

Oocytes are transcriptionally active during the growth phase, but transcription halts before oocyte maturation, at the prophase-arrested germinal vesicle stage, showing highly condensed chromatin surrounding the nucleolus (Clift and Schuh, 2013; De La Fuente et al., 2004). It is not until after fertilization that transcription fully resumes. It was initially accepted that embryonic genome activation would start between the four- and eight-cell stage in human preimplantation development (Braude et al., 1988) although recent evidence suggests that it initiates at the one-cell stage (Asami et al., 2022). The timing of transcriptional repression in GV oocytes is an important developmental transition to enable oocyte's full developmental potential (De La Fuente et al., 2004). The global silencing of transcription is accomplished due to chromatin modification, such as methylation and deacetylation of histones and DNA methylation (He et al., 2021). Chromatin in the oocyte nucleus changes from a diffuse or decondensed configuration, found predominantly in growing diplotene stage oocytes, and becomes progressively condensed around the nucleolus upon completion of oocyte growth (De La Fuente et al., 2004). Post-transcriptional control of gene expression ensures the storage and timely activation of maternal factors necessary for the oocyte-to-embryo transition. Noteworthy, transcripts are stored during oocyte growth to support oocyte maturation and are subsequently selectively degraded during maturation (Su et al., 2007). Since transcription is postulated to be absent during oocyte maturation, post-transcriptional regulation of mRNAs plays an essential role in the process. Immature oocytes contain transcripts with shorter poly-adenylated tails, which are stored in subcortical aggregates in the cytoplasm, ready for subsequent reactivation (Clift and Schuh, 2013). Following the onset of oocyte maturation, polyadenylation of dormant transcripts occurs in the cytoplasm, activating these mRNAs and enabling them to be translated (Clift and Schuh, 2013; Liu et al., 2023). Epigenetic modifications play a central role in regulation of gene expression. The histone modifications H3K9 and H3K4 trimethylation are well-characterized epigenetic marks that have been shown to be associated with transcriptional silencing in oocytes (Andreu-Vieyra et al., 2010; Chousal et al., 2018). Besides histone modifications, the oocyte acquires a unique DNA methylation pattern during oocyte growth, in which an increase in global levels of DNA methylation correlates with chromatin accessibility and active transcription (Qian and Guo, 2022). DNA methylation is catalysed by the DNA methyltransferase (DNMT) protein family, which is responsible for adding a methyl group to the fifth carbon of cytosine bases, predominantly in CpG dinucleotides, yielding a 5-methylcytosine (5 mC) (Portela and Esteller, 2010). 5 mC can be oxidized by the action of the ten-eleven translocation (TET) protein family, namely, TET1, TET2, and TET3, resulting in 5-hydroxymethylcytosine (5hmC), which is implicated in the active DNA demethylation mechanism (Branco et al., 2011). In human oocytes, studies using single-cell whole-genome bisulfite sequencing showed no differences in the global CpG methylation between GV to MII stage, although localized changes still occur throughout maturation (Yu et al., 2017). Noteworthy, non-CpG methylation levels increase from GV to MII stages, being associated with gene expression changes during maturation and directed by DNMT3B (Yu et al., 2020). Intriguingly, oocytes present a bimodal pattern of DNA methylation, i.e., methylation is largely restricted to actively transcribed regions, with intergenic or transcriptionally inactive regions showing low levels of methylation (Demond and Kelsey, 2020). Furthermore, transcription and chromatin accessibility correlate with *de novo* DNA methylation in growing oocytes (Yan et al., 2021), with transcription being required for the establishment of DNA methylation at imprinted genes in mouse oocytes (Chotalia et al., 2009).

The study of the oocyte transcriptome during maturation is of great relevance, as gene expression during their growth affects the quality of

the oocytes (Fan et al., 2015). Studies have shown that non-competent oocytes have a different transcriptional state compared to competent oocytes (Yanez et al., 2016). Thus, a comprehensive knowledge of the oocyte transcriptome is important for assisted reproductive technologies (ART), particularly the potential to improve *in vitro* maturation protocols, since it has been observed that *in vitro* matured oocytes express different genes compared to *in vivo* matured oocytes (Jones et al., 2008; Zhao et al., 2019). The transcriptome of oocytes has initially been studied using microarrays (Jones et al., 2008; Kocabas et al., 2006). With the development of Next-Generation Sequencing (NGS) techniques to analyse transcriptomes at a single cell level, it has been possible to study the oocyte transcriptome in several mammalian species (Tang et al., 2010); however, the study in humans is still limited, especially in *vivo* matured MII oocytes. In this study, we analysed the transcriptome of human oocytes at three different maturation stages, using single-oocyte RNA-Seq, with the aim of providing a transcriptomic profile and identifying key genes driving oocyte maturation.

2. Materials and methods

2.1. Single oocyte collection

Human oocytes analysed in this prospective study were collected from surplus material of oocyte donation programs from Assisted Reproduction Centres in Porto, Portugal (CRGAB - Centre for Reproductive Genetics Professor Alberto Barros and PROCRIAR Fertility Clinic), after obtaining informed consent from the donors. Characterization of all the oocyte donors is presented in Supplemental Table S1, with no statistically significant differences observed regarding Age, Body Mass Index (BMI) or Smoking status. All biological materials were donated voluntarily, anonymously and carried no personal identifiers. A routine genetic analysis of karyotype and the most frequent pathogenic variants for cystic fibrosis, spinal muscular atrophy and fragile-X was performed in all the donors, with the presence of a pathogenic/likely-pathogenic variant being an exclusion criterion. This study was approved by the institutional review board (Ethics Committee for Health of Hospital Sao Joao/Faculty of Medicine of the University of Porto -project reference CE 429-19) and conducted in accordance with the Declaration of Helsinki.

Oocyte donors underwent controlled ovarian hyperstimulation with a gonadotropin-releasing hormone (GnRH) antagonist protocol (0.25 mg, ganirelix, Orgalutran, MSD, Hertfordshire, UK). For stimulation, recombinant follicle-stimulating hormone was used (mostly with rFSH, follitropin alfa, Gonal-F; Merck Serono, Geneva, Switzerland and occasionally with Elonva; Organon, Kloosterstraat, Netherlands). For induction of maturation, a GnRH agonist (0.2 mg, triptorelin, Decapeptyl; Ipsen Pharma Biotech, Signes, France) was administered 36h before ultrasound-guided transvaginal oocyte retrieval.

The size of the follicles was monitored over several days before the follicle puncture, which allowed the optimal time for the puncture to be established (Supplemental Table 3). Therefore, different sizes of follicles were aspirated in puncture. Oocyte handling was performed in Origio media (Origio, Maløv, Denmark) in a heated airflow (K-system, Kivex Biotech, Denmark; Cooper Surgical, Maløv, Denmark). Denudation was performed enzymatically with Cumulase (Origio) and then mechanically in SPM (Origio) with Handling pipettes (Vitrolife). After denudation, oocytes were placed in a tube with 3 µL of FERT medium (Origio) and immediately stored at -20 °C for a maximum of 2 h. The oocytes are then snap-frozen and incubated at -80 °C after adding 2.5 µL of Buffer RLT (Qiagen).

Human oocytes were classified into three distinct maturation stages, according to their morphological characteristics, namely, the presence of an intact and visually distinct nuclear membrane and prominent nucleolus (Germinal Vesicle - GV), the presence of an extruded first polar body (Metaphase II - MII) and a homogeneous cytoplasm (Metaphase I - MI) (Fig. 1A). Abnormal-appearing oocytes, with signs of

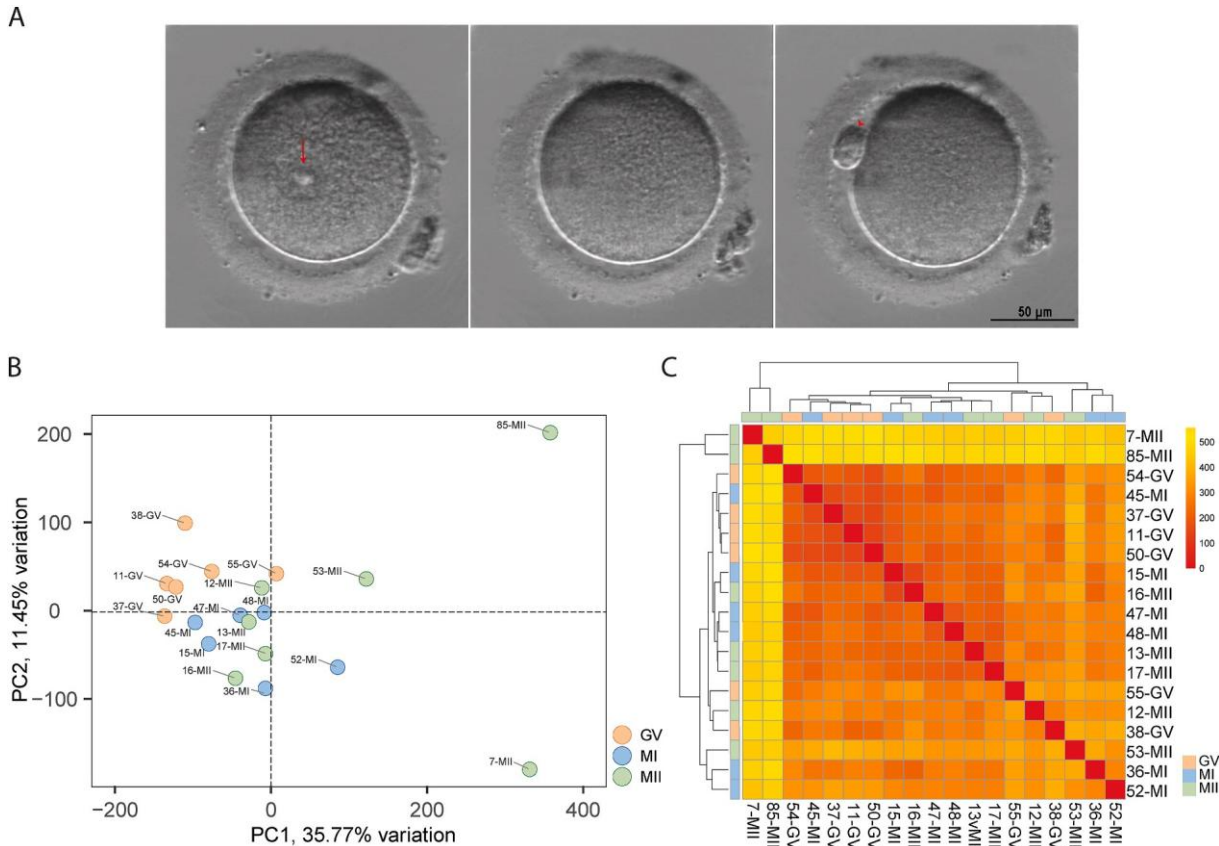


Fig. 1. – Characterization of human oocyte maturation stages. A) Phase-contrast microscopy images of human oocytes at each maturation stage: GV – Germinal-Vesicle; MI – Metaphase I; MII – Metaphase II. Arrow – nuclear envelope in GV oocyte; Arrowhead – first polar body in MII oocyte. B) Principal Component Analysis (PCA) plot of all oocytes. GV oocytes are represent in orange, MI oocyte in blue and MII in green and C) Heatmap showing the correlation coefficients between transcriptomes of all oocytes, reflecting similarities and differences in gene expression across the maturation stages. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

degeneration, were excluded from this study.

2.2. Library preparation for RNA-Seq analysis

To limit the influence of external factors, transcriptome analysis was performed in oocytes collected from a single IVF clinic (CGRAB). A total of 19 oocytes were individually collected, 6 in the Germinal Vesicle stage (Prophase I of the cell cycle; GV), 6 were in Metaphase I (MI) and 7 were in Metaphase II (MII). These oocytes were retrieved from eleven oocyte donors and from only one cycle of ovarian stimulation per donor. Detailed information about the oocytes donors and follicle size before ovarian puncture is provided in [Supplemental Tables S1 and S2](#). Full-length single-oocyte RNA-seq (soRNA-seq) libraries were prepared at the Genomics Unit of Gulbenkian Institute of Science (IGC, Portugal), following the protocol described by Macaulay and collaborators ([Macaulay et al., 2016](#)). Each single oocyte, collected in RLT reagent (Qiagen), was processed for cDNA synthesis, as described in the original protocol. Quality control of the synthesized cDNA was performed using Fragment Analyzer (Agilent Technologies). Library preparation including cDNA ‘tagmentation’, PCR-mediated adaptor addition and amplification of the adaptor-ligated libraries was performed following the Nextera library preparation protocol (Nextera XT DNA Library Preparation kit, Illumina), as described by Baym and collaborators ([Baym et al., 2015](#)). Amplified libraries were analysed by Fragment Analyzer (Agilent Technologies) and then sequenced in a NextSeq 2000 (Illumina Inc) using 100 SE P2 to an average sequencing depth of 20 million reads per cell. Sequence information was extracted in FastQ format, using Illumina DRAGEN FASTQ Generation v3.8.4.

2.3. Sequencing data processing

Initial quality check of raw sequencing data was performed using FastQC (Babraham BioInformatics) (Version 0.12.0) and was considered of good quality (99% > Q30). Reads were then mapped to the human genome (GRCh38) with Hisat2 v2.2.2 ([Kim et al., 2015](#)). Gene expression quantification was carried out using FeatureCounts (Version 2.0.3) ([Liao et al., 2013](#)) and Kallisto (Version 0.50.0) ([Bray et al., 2016](#)) using gene annotations from Ensembl version 106 (April 2022) in order to compare and validate the results obtained with the two bioinformatic tools. Expression values were normalized to CPM (Counts Per Million) using the Trimmed Mean of the M-Values (TMM) approach ([Robinson and Oshlack, 2010](#)) as available in the edgeR (Version 3.32.1) package from the R software (Version 4.0.5).

2.4. Bioinformatic analysis

Principal component analysis (PCA) was performed with normalized CPM values to assess the overall similarity between samples, using the R package PCAtools (Version 2.2.0). R (Version 4.0.5) was also used to plot a heatmap representing similarity between samples using the Euclidean distance of normalized gene expression. The expressed genes were determined considering the normalized CPM value, which should be greater than 1 (or log2CPM greater than 0) in all samples of the group. Genes uniquely expressed in each phase were defined as genes whose normalized CPM value was greater than 1 in all samples from only one of the stages. Differentially Expressed Genes (DEGs) were identified by comparing all oocyte maturation stages (GV vs MI; GV vs MII; MI vs MII). DEGs were identified using the quasi-

likelihood F-test (Lun et al., 2016) as available in the edgeR (Version 3.32.1) package in R (Version 4.0.5). Genes with $FDR \leq 0.05$ and $Fold\text{-}Change (FC) > |2|$ were assigned as differentially expressed. As an alternative and less restrictive tool, DESeq2 (Version 1.30.1) (Love et al., 2014) was also utilized to extract DEGs, using a p adjusted value < 0.05 and $\log_2 FC > |1|$.

KEGG pathway (Kyoto Encyclopedia of Genes and Genomes; <http://www.genome.jp/kegg/>) and Gene ontology (GO) analysis (<http://geneontology.org>) was performed using the tool g:GOST provided by GProfiler (Raudvere et al., 2019) (Version e111_eg58_p18_f463989d). GO terms with an adjusted p -value < 0.05 were considered significant. Venn Diagrams were constructed using the VIB/UGent Bioinformatics and Evolutionary Genomics tool - <https://bioinformatics.psb.ugent.be/webtools/Venn/> and jvenn - <https://jvenn.toulouse.inrae.fr/app/index.html> (Bardou et al., 2014).

Volcano plots were plotted to visualize the differentially expressed genes in each comparison using the package EnhancedVolcano (Version 1.8.0) from R. Violin plots were designed using R software and ggplot2 package (Version 3.3.6).

2.5. Gene expression analysis by quantitative RT-PCR (qRT-PCR)

RNA extraction from human oocytes was performed using Power SYBR® Green Cells-to-Ct™ Kit (Invitrogen), allowing reverse transcription (RT) and real-time PCR directly from cell lysates. Briefly, we added 50 μ L of lysis solution (with DNase I) to the single oocyte and incubated at RT for 5 min. Then, 5 μ L of stop solution were added to the lysate and incubated for 2 min at RT. After, 10 μ L of the lysate were used for the reverse transcription reaction and 2 μ L of the cDNA was used for the RT-qPCR, using PowerUp™ SYBR™ Green Master Mix. Gene expression was evaluated using a DNA-binding dye, $2 \times$ PowerUp SYBR Green Master Mix (Thermo Fisher Scientific), following the manufacturer's instructions. Specific primers were designed for amplification of *DNMT1*, *ZFAND2A* and *UBB* as reference gene (Supplemental Table S4). We analysed the expression of the following genes: *DNMT1* in 9 GV oocytes and 4 MII oocytes, *ZFAND2A* in 10 GV oocytes and 3 MII oocytes. Statistical analysis was performed using the Mann-Whitney U test, with the Statistical Package for Social Sciences (SPSS, IBM) software v29.0.

4. Results

4.1. Transcriptome analysis of human oocytes reveals a higher number of expressed and uniquely expressed genes in GV oocytes, comparing to MI and MII oocytes

We isolated single human oocytes that were classified according to their morphological characteristics into three distinct stages: Germinal Vesicle (GV), Metaphase I (MI) and Metaphase II (MII) (Fig. 1A), as described in the Material and Methods section. Then, we performed transcriptomic analysis through single-oocyte RNA-Seq (soRNA-Seq). A total of 19 oocytes, originating from 11 donors, were individually sequenced (6 GV, 6 MI and 7 MII). The number of mapped and aligned reads for each sample is shown in Supplemental Table S3.

Principal component analysis (PCA), after quantification of gene expression using FeatureCounts (Liao et al., 2013), showed relatively dispersed samples but a degree of aggregation based on the oocyte maturation stage was observed, particularly in GV oocytes, which appear predominantly on the upper left quadrant and more distant to the other two stages (Fig. 1B). The group of MI oocytes might include oocytes that are transitioning to MII but have not yet undergone polar body extrusion, hence appearing more closely related to MII oocytes. MII oocytes showed less aggregation in the PCA plot as well as in the distance heatmap (Fig. 1C), which might reflect more heterogeneity of the samples. When using kallisto (Bray et al., 2016) as an alternative tool for gene expression quantification, a PCA plot with the same distribution

was obtained as well as a similar heatmap (Supplementary Figs. S1 and S2).

In order to identify which genes were expressed at each maturation stage, we considered transcripts with a CPM (Counts Per Million) value greater than 1 (or $\log_2 CPM > 0$) in all the samples belonging to each group, retrieving a number of expressed genes of 9660 in GV, 8734 in MI and 5889 in MII oocytes (Supplementary File S1). The top 10 most highly expressed genes at each maturation stage are shown in Fig. 2A. Of these, *TUBB8* (Tubulin Beta 8 Class VIII) was the most expressed gene at all maturation stages, along with other genes of the same family (*TUBB8P7* and *TUBB8B*). Mitochondrial genes such as *MT-ND4* and *MT-CO1* were also highly expressed in all maturation stages, as well as the Histone *H3-3B*. Examples of other genes that were highly expressed at all stages were *DPPA3* (Developmental Pluripotency Associated 3), *WEE2* (WEE2 Oocyte Meiosis Inhibiting Kinase), *OOSP2* (Oocyte Secreted Protein 2), all *ZP* (Zona pellucida) genes, *BMP15* (Bone Morphogenetic Protein 15) and *GDF9* (Growth differentiation factor 9) (Fig. 2A and Supplementary File S1).

Gene ontology analysis of the highly expressed genes ($\log_2 CPM > 10$) in each maturation stage revealed similar terms for all three oocyte stages, namely terms related to mitochondrial function and oocyte development and maturation (Supplementary Fig. S3).

Further analysis revealed that, of the genes expressed in each maturation stage, 5602 were common to all three stages whereas 1328 were unique in GV, 401 in MI and 72 in MII (Fig. 2B and Supplementary File S2). GV and MI shared the highest number of genes ($n = 2623$) whereas MI and MII, and GV and MII, shared a similar number of genes ($n = 108$ and 107, respectively) (Fig. 2B).

Gene Ontology analysis of the 5602 genes that were expressed in all three maturation stages revealed terms related with organelle organization, metabolic and biosynthetic processes as well as cell cycle (Fig. 2C). Regarding molecular function, protein binding was the most significant term, followed by catalytic activity and enzyme binding. Concerning the uniquely expressed genes and in particular for molecular function, protein binding was the most significant term in GV oocytes followed by catalytic activity, ion binding and transferase activity (Supplemental Table S5). Interestingly, we also obtained the Molecular Function terms “S-adenosylmethionine-dependent methyltransferase activity” and “methyltransferase activity”, possibly related to epigenetic reprogramming events occurring in oocyte development (Qian and Guo, 2022).

Regarding uniquely expressed genes in MI ($n = 401$), we only obtained two statistically significant GO terms which were “biological regulation” and “regulation of biological process” (Supplementary Table S5). No statistically significant GO terms were found for unique genes from MII oocytes, possibly due to the low number of genes analysed.

4.2. Differential expression analysis reveals higher differences between GV and MII maturation stages

In order to identify transcriptomic changes during oocyte maturation, we extracted Differentially Expressed Genes (DEGs) with fold change ($FC > |2|$) (or $\log_2 FC > |1|$) and $FDR < 0.05$ (Supplementary File S3). Using EdgeR (Lun et al., 2016), we identified 42 DEGs between GV and MI and 1165 DEGs between GV and MII stages; no DEGs were detected between MI and MII maturation stages. On the other hand, using DESeq2 (Love et al., 2014) as an alternative method to extract DEGs, we obtained 403 DEGs between GV and MI, 2981 DEGs between GV and MII and 80 DEGs between MI and MII, with a p adjusted value < 0.05 and $\log_2 FC > |1|$. Comparison of all the bioinformatic tools utilized revealed EdgeR to be a more stringent tool, capturing less DEGs than DESeq2. Importantly, nearly all EdgeR DEGs were also present in DESeq2 analysis (Supplementary Fig. S4); hence, we proceeded further analyses with the DEGs obtained by EdgeR.

Of the 42 DEGs detected between GV and MI stages, 38 were

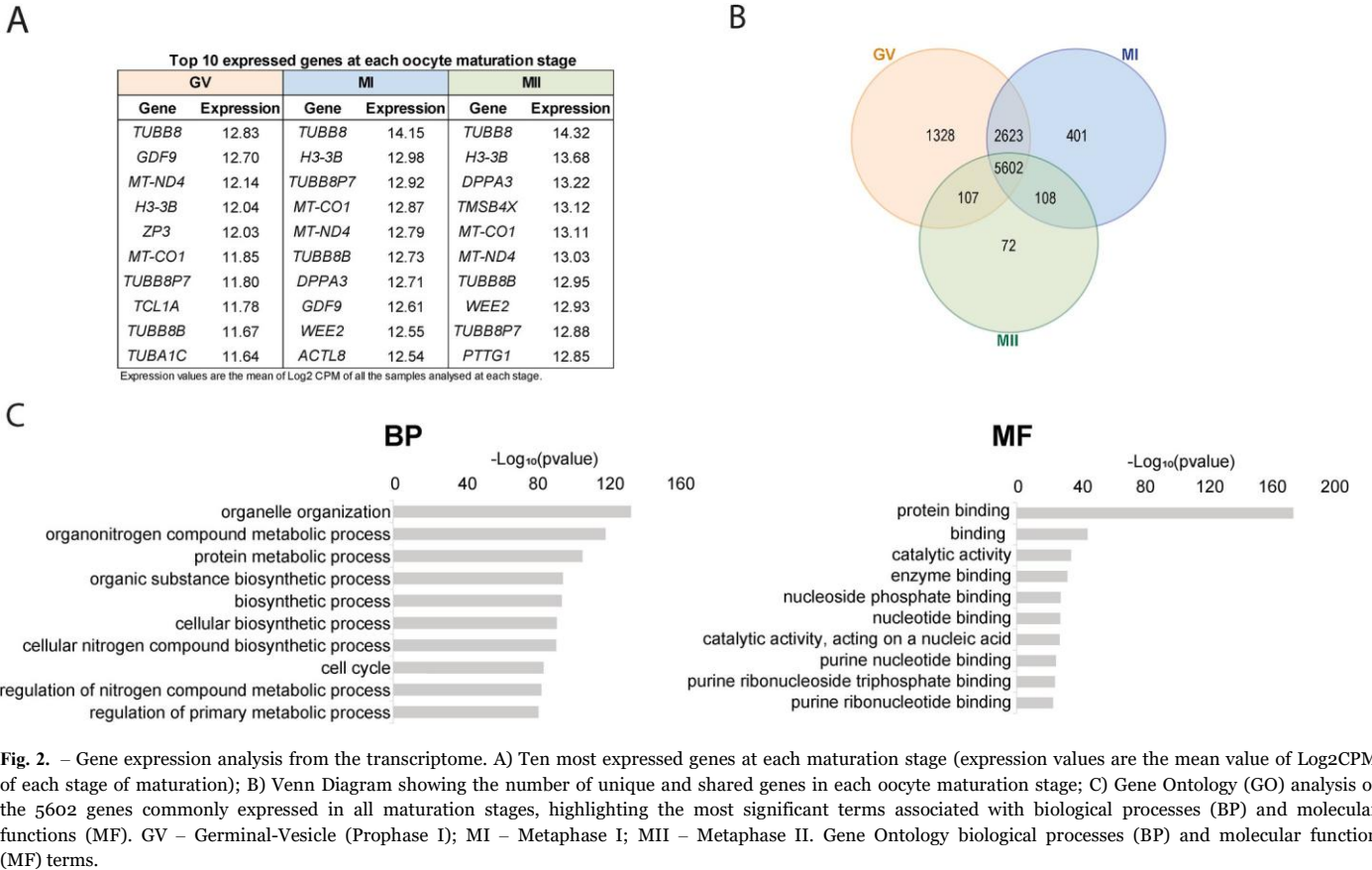


Fig. 2. – Gene expression analysis from the transcriptome. A) Ten most expressed genes at each maturation stage (expression values are the mean value of Log2CPM of each stage of maturation); B) Venn Diagram showing the number of unique and shared genes in each oocyte maturation stage; C) Gene Ontology (GO) analysis of the 5602 genes commonly expressed in all maturation stages, highlighting the most significant terms associated with biological processes (BP) and molecular functions (MF). GV – Germinal-Vesicle (Prophase I); MI – Metaphase I; MII – Metaphase II. Gene Ontology biological processes (BP) and molecular function (MF) terms.

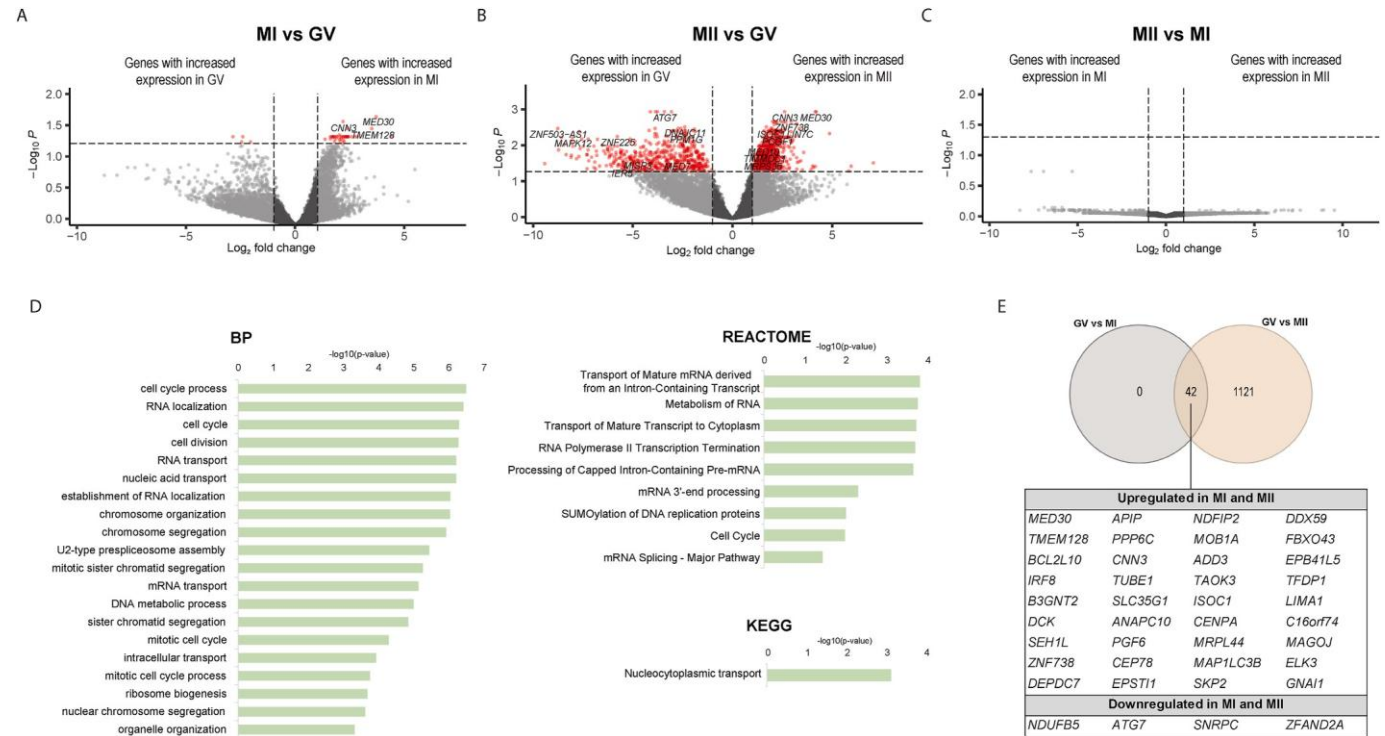


Fig. 3. – Differentially Expressed Genes during oocyte maturation. DEGs were defined as $FDR \leq 0.05$ and $FoldChange (FC) > |2|$. Volcano Plots for comparison between A) MI and GV oocytes B) MII and GV oocytes C) MII and MI oocytes. D) Functional enrichment analysis, displaying the significant Gene Ontology (GO) biological processes (BP), REACTOME pathways, and KEGG pathways associated with upregulated DEGs in MII relative to GV oocytes. E) Venn diagram of DEGs obtained from the comparisons between MI vs. GV and MII vs. GV, showing the overlap and specific DEGs. GV – Germinal-Vesicle; MI – Metaphase I; MII – Metaphase II.

upregulated in MI oocytes and 4 were downregulated (Fig. 3A and Supplementary File S3). GO analysis of the MI upregulated genes revealed an enrichment of the term “mitotic cell cycle” (Supplementary File S3).

Regarding the comparison between GV and MII oocytes, we identified a total of 1165 DEGs, of which 635 were upregulated and 528 were downregulated in MII oocytes (Fig. 3B and Supplementary File S3). GO analysis of the upregulated genes in MII revealed relation to cell cycle processes, RNA localization and transport, chromosome organization and segregation (Fig. 3D and Supplementary File S3). Downregulated genes were related to nucleoplasm and proteasome activity (Supplemental File S3). Noteworthy, all the 42 DEGs identified in the GV vs MI comparison were also present in the GV vs MII comparison, whereas 1121 DEGs were unique to the GV vs MII comparison (Fig. 3E).

Interestingly, no DEGs were identified between MI and MII oocytes, reflecting a similar transcriptional state between oocytes in these two meiotic divisions (Fig. 3C).

Expression plots of some of the DEGs are shown in Fig. 4; *WEE2* and *DPPA3*, two of the top10 highly expressed genes (Fig. 2A), are upregulated throughout oocyte maturation, as well as *CDC20*, which encodes a protein that is a co-activator subunit of anaphase-promoting complex/cyclosome (APC/C) and is crucial for oocyte maturation (Sang et al., 2021).

Examples of downregulated genes are *PANX1* and *PATL2*, which have been implicated in oocyte death and GV arrest, respectively (Sang et al., 2021). *PANX1* encodes pannexin 1, a glycoprotein that has an important role in cellular communication, namely for the transport of adenosine 5'-triphosphate (ATP). Variants in *PANX1* may lead to aberrant ATP release followed by oocyte death (Wang et al., 2021). *PATL2* is an RNA-binding protein that may play the role of translational repressor and biallelic mutations in *PATL2* may lead to oocyte GV arrest, and, therefore, women's infertility (Chen et al., 2017). Another important gene which is also downregulated throughout oocyte maturation is the *ZAR1* (Zygote Arrest 1), in particular for coordination of maternal mRNAs storage, translation and degradation (Rong et al., 2019).

MED30, *ZFAND2A* and *ZNF738* are three of the most significant DEGs, with *MED30* and *ZNF738* being upregulated and *ZFAND2A* being downregulated in MII (confirmed by qRT-PCR in an independent set of samples; Suppl Fig. S5).

4.3. Expression of epigenetic regulators throughout human oocyte maturation

Epigenetic modifications play a pivotal role during oocyte meiotic maturation. Hence, we focused our analysis on the expression of several epigenetic regulators (Fig. 5 and Supplemental File S4). Histone

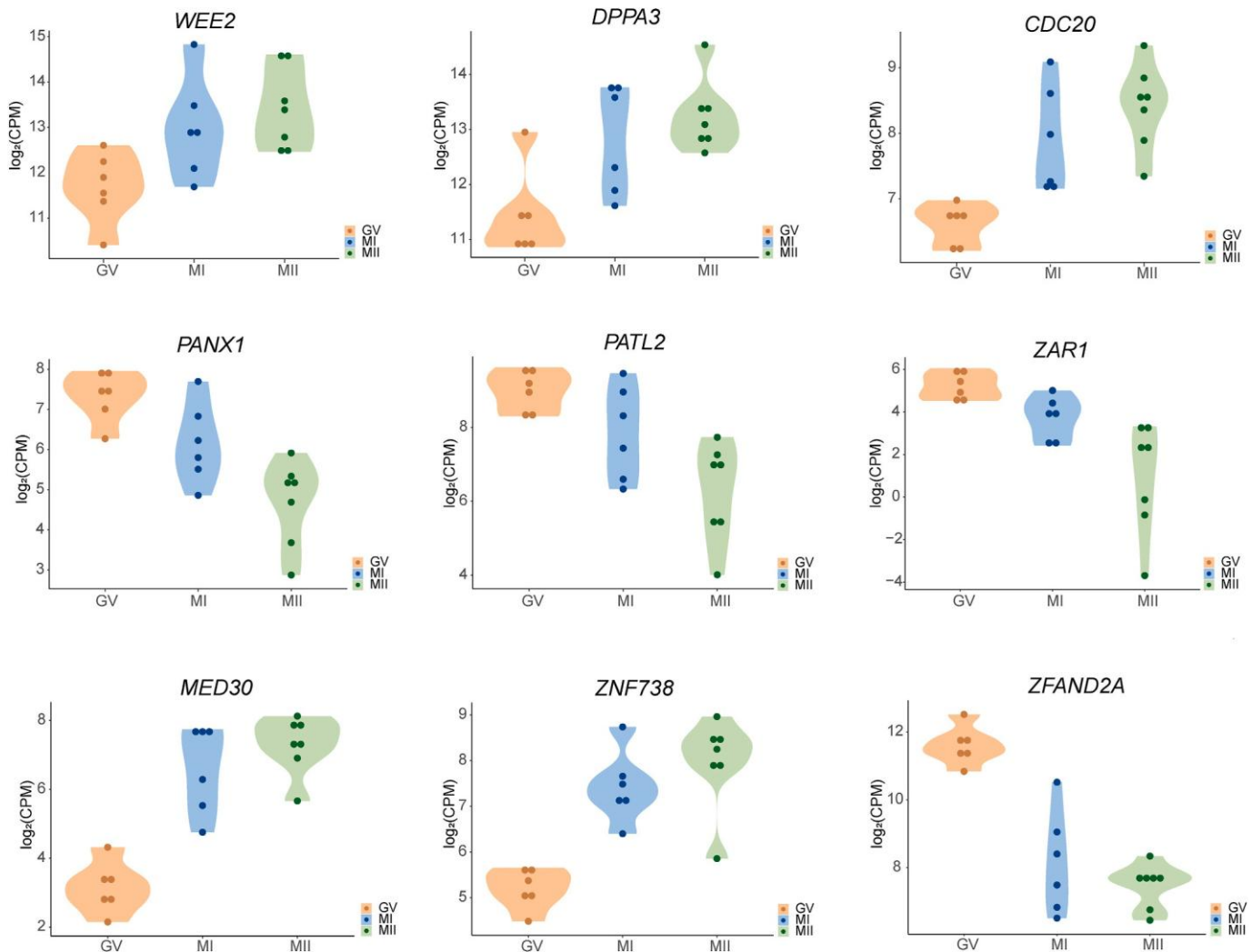


Fig. 4. – Violin plots of Differentially Expressed Genes (DEGs) during oocyte maturation. Violin plots illustrate the expression levels of selected DEGs with FDR ≤ 0.05 and absolute Fold Change (FC $> |2|$), specifically for the genes: *WEE2*, *DPPA3*, *CDC20*, *PANX1*, *PATL2*, *ZAR1*, *MED30*, *ZNF738* and *ZFAND2A*. Orange: GV oocytes, Blue: MI oocytes, Green: MII oocytes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

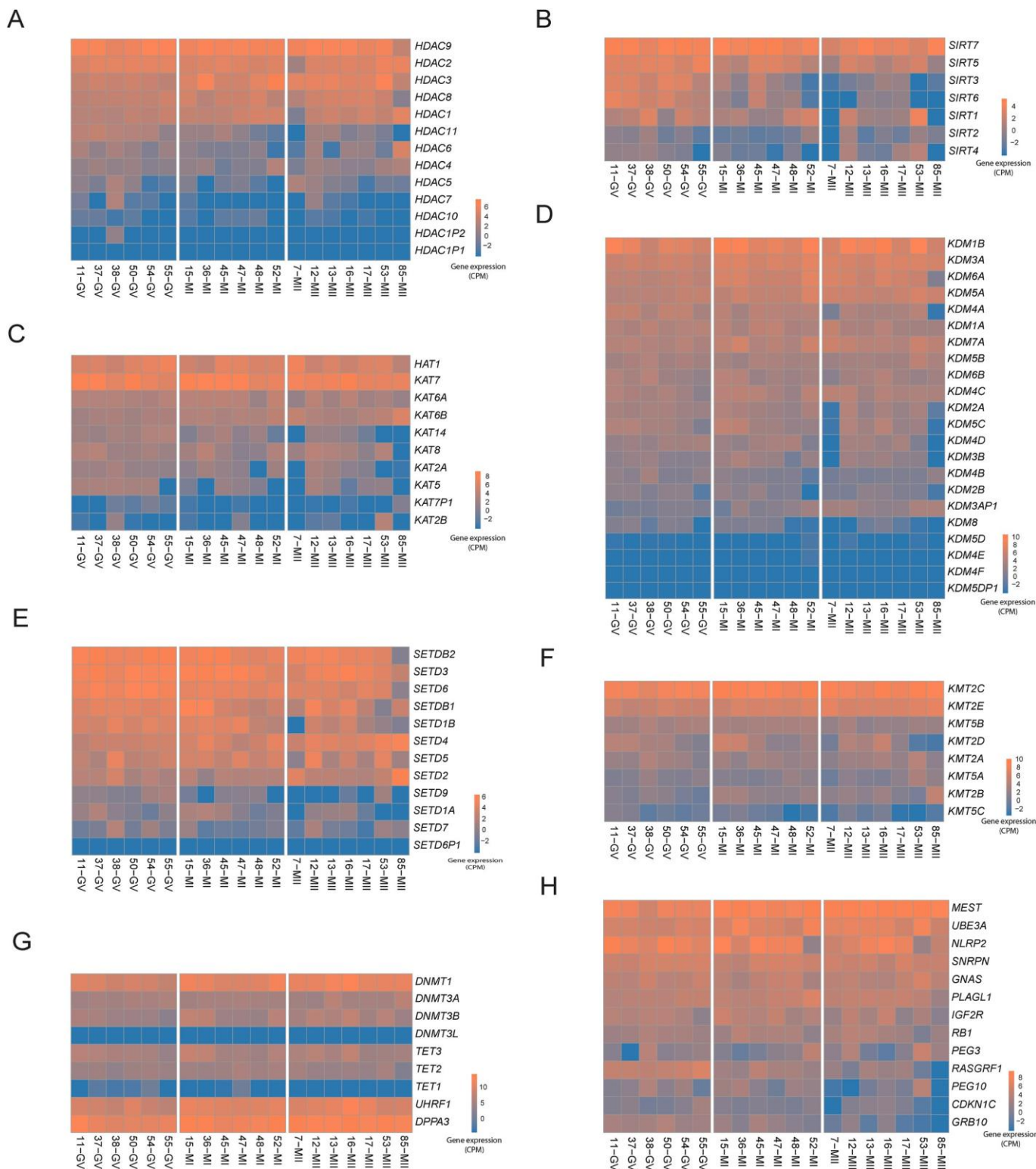


Fig. 5. Heatmaps of epigenetic regulators and imprinted genes. Histone deacetylases (A) and Sirtuins (B); Histone acetyltransferases (C); Histone demethylases (D); SET-domain protein methyltransferases (E); Histone Methyltransferases (F); DNA methyltransferases and co-factors and Demethylases (G); Imprinted genes (H). The color gradient represents the level of gene expression (in CPM). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

deacetylases (HDACs), especially *HDAC9*, *HDAC2* and *HDAC3* were highly expressed in all oocyte maturation stages (Fig. 5A). Sirtuins (SIRTs) are nicotinic adenine dinucleotide (+)-dependent histone deacetylases (Wu et al., 2022). Whereas the most expressed Sirtuins throughout all the maturation stages were *SIRT7* and *SIRT5* (Fig. 5B),

SIRT3 and *SIRT6* showed differential expression, being downregulated in MII oocytes (Supplemental File S3).

Regarding histone acetyltransferases, *HAT1* and *KAT7* were the most highly expressed throughout meiotic maturation (Fig. 5C). Other histone-modifying enzymes that were highly expressed in all stages were

lysine demethylases (KDM), particularly *KDM1B* and *KDM3A* (Fig. 5D). Of all the KDMs, only *KDM3A1* was identified as a DEG showing increased expression in MII oocytes (Supplemental File S3).

When we examined histone methyltransferase enzymes, such as the SET domain (SETD) and histone lysine methyltransferase (KMT), we observed that, in general, *SETD* exhibited higher expression levels in the three maturation stages (Fig. 5E). However, it was the *KMT2C* gene that showed the highest expression (Fig. 5F). Additionally, of all the histone methyltransferases, only *KMT2E* was identified as a DEG, showing upregulation in MII oocytes (Supplemental File S3).

Regarding DNA methylation writers, *DNMT1* (DNA methyltransferase 1) showed high expression during oocyte maturation and upregulation in MII oocytes (confirmed by qRT-PCR in an independent set of samples; Suppl Fig. S5), together with its recruiter *UHRF1* and binder *DPPA3* (Fig. 5G). Of the TET-family of enzymes, which play a role in the active DNA demethylation process, *TET3* was the most highly expressed, consistent with previous observations in the mouse oocyte and its role in the conversion of 5 mC to 5hmC in the early zygote (Wossidlo et al., 2011). Regarding imprinted genes, we observed a high expression of mesoderm-specific transcript (*MEST*), Ubiquitin Protein Ligase E3A (*UBE3A*) and NLR Family Pyrin Domain Containing 2 (*NLRP2*) genes in all stages of oocyte maturation (Fig. 5H). Additionally, we observed that *MEST* was upregulated in MII oocytes whereas Ras Protein Specific Guanine Nucleotide Releasing Factor 1 (*RASGRF1*) was downregulated (Supplemental File S3).

5. Discussion

In this study, we performed transcriptomic analysis of human oocytes in three developmental stages of the meiotic maturation process. The quality of oocytes and embryos in the IVF lab is determined by morphological markers, whereas there is a lack of molecular markers for determining oocyte quality (Sang et al., 2021). Our results describe the gene expression patterns in each stage of oocyte maturation, with the number of genes expressed showing a reduction from GV to MI and subsequently from MI to MII, which is consistent with RNA degradation observed during oocyte maturation (Jiang and Fan, 2022). This decrease in the number of expressed genes during oocyte maturation has been documented by other authors (Llonch et al., 2021; Takeuchi et al., 2022; Yan et al., 2021; Yu et al., 2020). Indeed, in a recent meta-analysis including human oocyte transcriptomic data from 6 independent studies, a 30% decline in total mRNA from GV to MII stage was described (Ducreux et al., 2023). Interestingly, we found that the number of unique genes, i.e., genes that were only expressed in one of the oocyte stages, also decreased from GV to MII, as observed by others (Yu et al., 2020), albeit the number of unique genes we observed is substantially higher than the number reported by Yu and collaborators. Furthermore, in our study, 72 genes are uniquely expressed in MII stage, and 635 genes are upregulated during oocyte maturation, from GV to MII stages, reinforcing the idea that transcriptional regulation occurs throughout meiotic divisions. As proposed by Ducreux and colleagues, this may reflect the amount of mRNAs that can be recruited for translation and therefore have not been targeted for degradation due to shorter polyA tails. It could also result from polyA tail lengthening during the GV to MII transition, leading to their upregulation in MII oocytes (Ducreux et al., 2023).

We obtained a high number of differentially expressed genes when comparing GV and MII oocytes, some of which are known to be relevant for oocyte maturation. In fact, of these DEGs, 148 were also observed by Pietroforte and collaborators (Pietroforte et al., 2023), of which we highlight some that were deemed to be relevant for the maturation of the oocyte, such as the *ZARI*, *PATL2*, *TRIP13*, *RBBP7* and *WEE2*. Additionally, *ZARI*, *PATL2*, *RBBP7* and *WEE2* were also identified as differentially expressed genes by Yu and collaborators, between MII oocytes and MI oocytes (Yu et al., 2020), highlighting the robustness of the results across different laboratories and studied populations.

Regarding the expression of specific genes, *TUBB8* was the most highly expressed gene across all three stages. *TUBB8* encodes a primary beta-tubulin subunit of oocytes and early embryos and has a key role in meiotic spindle assembly and maturation in human oocytes; it has been shown that mutations in *TUBB8* result in maturation arrest at meiosis I, by disrupting microtubule behaviour and oocyte spindle assembly, leading to female infertility (Feng et al., 2016). *TUBB8B* and *TUBB8P7* were also highly expressed in all three stages; *TUBB8B* is a paralog of *TUBB8* and a protein coding gene whereas *TUBB8P7* is a pseudogene. Several mitochondrial genes were also highly expressed in all maturation stages, consistent with the reported abundance of mitochondria in oocytes; *MT-ND4* and *MT-CO1* were the most highly expressed. Another gene that was also observed to be highly expressed was the gene *H3-3B* that codes for histone variant H3.3, which was shown to be essential for paternal chromatin remodelling after fertilization and for the subsequent paternal genome activation during embryogenesis (Kong et al., 2018). Other highly expressed genes of note are *DPPA3* (a.k.a. STELLA), which is essential for female fertility and encodes a protein that plays an important role in shaping the oocyte methylome, preventing *de novo* methylation by UHRF1-mediated DNMT1 (Li et al., 2018). Additionally, *WEE2* and *OOSP2*, are critical for meiosis and, therefore, for oocyte maturation (Hu et al., 2022; Sang et al., 2018). *BMP15* and *GDF9* encode for two oocyte-derived factors that regulate granulosa cell proliferation and differentiation and induce antrum formation (Alan and Miyano, 2020).

Regarding the differential expression throughout oocyte maturation, we observed that genes involved in cell cycle, RNA processes and epigenetic regulation were upregulated in MII oocytes, as noted by Ducreux and collaborators (Ducreux et al., 2023) whereas, in our study, the MII downregulated genes were related to proteasome complex, indicating that maternal protein degradation occurs predominantly before MII stage, being inhibited or halted during the MII oocyte arrest (Karabinova et al., 2011).

Interestingly, *DNMT1*, which is considered a maintenance DNA methyltransferase, i.e., copying with high fidelity the methylation patterns during DNA replication in the S-phase of the cell cycle, is highly expressed throughout oocyte maturation, including across meiotic divisions, being significantly upregulated in MII oocytes, as observed by others (Yu et al., 2020). We have previously observed high expression of *DNMT1* also during meiotic divisions of human spermatogenic cells (Marques et al., 2011), suggesting that DNMT1 might play a role in methylation repair to prevent methylation errors, particularly at imprinted genes, to be transmitted to the next generation. Indeed, DNMT1 has been suggested to act as a *de novo* methyltransferase upon its discovery (Yoder et al., 1997) and later shown to be regulated by *DPPA3* (a.k.a. Stella), safeguarding the unique oocyte epigenome (Li et al., 2018). *UHRF1* (a.k.a. NP95), which is believed to recruit *DNMT1* (Sharif et al., 2007), also had a high expression in all oocyte maturation stages.

Concerning DNA demethylation, two of the putative DNA demethylases, *TET2* and *TET3*, were also expressed in all oocyte maturation stages; in particular, *TET3* has been shown to be highly expressed in mouse oocytes and act upon the active DNA demethylation events observed in the paternal pronucleus of mouse zygotes (Gu et al., 2011; Wossidlo et al., 2011).

We consider that a strength of our work is that we performed transcriptomic analysis in single oocytes given that single-cell RNA sequencing can identify differences which were previously overlooked with bulk analysis (Wu, 2022); additionally, we analysed MII oocytes that underwent *in vivo* maturation, whereas several other studies performed *in vitro* maturation; furthermore, considering that each fertility clinic relies on their own gonadotropin stimulation protocol, another advantage of our study is that transcriptomic analysis was performed in human oocytes collected from a single fertility clinic. Concerning limitations, one possible shortcoming is that we utilized an oligo-dT primer for RNA capture and cDNA synthesis (Macaulay et al., 2016) which directs the transcriptome towards poly-A-tailed mRNAs. Nevertheless,

RNA-Seq protocols that capture only polyadenylated mRNAs have been utilized by other authors to analyse the human oocyte transcriptome (Llonch et al., 2021; Ntostis et al., 2021; Reyes et al., 2017; Takeuchi et al., 2022; Yu et al., 2020). As oocytes are postulated to be transcriptionally silent, changes in gene expression during maturation might be controlled by post-transcriptional mechanisms, such as cytoplasmic polyadenylation of stored mRNAs. mRNA Poly(A) tails, which promote translation and prevent degradation, undergo size changes throughout oocyte maturation, whereas proteins highly important for meiotic divisions have increased polyadenylation during maturation (Liu et al., 2023; Yang et al., 2020).

In conclusion, our study provides novel insights about gene expression changes specific to oocyte maturation, namely a marked reduction in the overall number of expressed genes from GV to MII stage, with a higher number of genes specifically expressed in MII stage oocytes. We also underscore a high expression of DNMT1 through maturation, we noted high expression of several mitochondrial genes, as well as, TUBB8 gene, a critical gene for meiotic spindle assembly, across all stages. Finally, our results show that genes involved in cell cycle regulation, RNA processing, and epigenetic control are significantly upregulated at the MII stage, while proteasome-related genes are downregulated, suggesting a shift in maternal protein degradation timing. Together, these findings expand our understanding of the molecular mechanisms underlying oocyte quality and maturation, contributing for greater understanding of oocyte quality and improvement of fertility treatments.

CRedit authorship contribution statement

Carla Caniçais: Investigation, Methodology, Visualization, Writing – original draft. **Daniel Sobral:** Data curation, Formal analysis, Software, Validation, Writing – review & editing. **Sara Vasconcelos:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Mariana Cunha:** Investigation, Methodology, Writing – original draft. **Alice Pinto:** Investigation, Methodology, Writing – original draft. **Joana Mesquita Guimarães:** Investigation, Methodology, Supervision, Writing – original draft. **Fátima Santos:** Formal analysis, Investigation, Methodology, Supervision, Visualization, Writing – original draft. **Alberto Barros:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft. **Sofia Dória:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **C. Joana Marques:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Data availability

The datasets and codes generated in this study were deposited in Zenodo (<https://doi.org/10.5281/zenodo.14163312>). All the other data are available from the corresponding author upon reasonable request.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ydbio.2024.12.004>.

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